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(54) Title: CONTROLLED GERMINATION USING INDUCIBLE PHYTATE GENE

(57) Abstract

The present invention provides a plant containing a phytic acid gene that is expressed in the seed of the plant only when desired. The present invention has two traits that can be induced or switched. The traits are (i) nongerminable seed and, (ii) low or no production of phytic acid.

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Controlled Germination Using Inducible Phytate Gene

The present invention is based on, and claims benefit to U.S. Provisional Application No. 60/055,323, filed August 11, 1997, the entire contents of which is incorporated herein by reference.

Field of Invention

The present invention provides a plant containing an inducible gene (phytate gene) that controls seed germinability by regulating phytic acid content as well as methods of making and using the plant, seed therefrom, and progeny thereof

Background

Low phytic mutants in maize are known. The age of recombinant DNA has recently become a financial success in the agricultural industries. Herbicide resistant and insect resistant cotton has been successfully marketed, as have soybeans and corn. Before transformed plants there were some crops that carried herbicide resistance due to mutations in the crops. For example, IT ™ corn produced by Garst Seed Company is a mutant plant that is herbicide resistant (Imazethapyr) HERBICIDE PURSUIT® (American Cyanamid).

Mutant plants have also been used to produce added value traits in a number of crops. For example, in maize, waxy, amylose extender and other mutants are used to produce special starch traits, and, in sweet corn, the sugary mutation is utilized.

A low phytic acid maize mutant, lpa1-R, has been produced by Raboy (U.S. Patent No. 5,689,054 (lpa1-1, described therein) . The commercial introduction of this lpa1 mutant is expected to occur in 1999. A number of

companies are presently testing this new low phytic acid mutant. A number of different mutants in maize and different species which carry this low phytic trait can be produced by the use of mutating agents and mutational methods of breeding corn.

Transposon tagging is a known method of gene identification and sequencing which involves mutating plants with a transposable element. As one advantage, this method provides a means of identifying and sequencing the gene associated with the mutation. Once a gene has been cloned it can be transferred between species. Methods of moving genes from one plant species to another plant species through the use of gene identification, sequencing and transformation are well known in the industry. Annu. Rev. Plant Physiol. Plant Molecular Biology 1992. 43: 49-82 by Virginia Walbot, entitled "Strategies for Mutagenesis and Gene Cloning Using Transposon Tagging and T- DNA Insertional Mutagenesis". A specific example of using transposon tagging employing the mu1 element for the genetic isolation, and cloning of an amylose extender gene in maize was reported in the Plant Cell, Vol. 5, pp.1555-1566, (November 1993) by Philip Stinard et al, entitled "Genetic Isolation, Cloning and Analysis of a Mutator-induced, Dominant Antimorph of the Maize Amylose Extender1 Locus." This article reports mutant plants subjected to transposon tagging and the identification of the mutated gene. The gene was sequenced by methods known in the art. The sequenced portion was used as a probe to identify the mutant gene, the gene was then cloned. Cloning of genes has been performed and is well established as a technique. Techniques of DNA recombination used throughout this invention are known to those skilled in the art and are described in Maniatis et al., MOLECULAR CLONING: a Laboratory Manual, Cold Spring Harbor Laboratory , Cold Spring Harbor, N.Y.)

Switchable or inducible transformation constructs are known and may be made by means known in the art. Often, after a gene is cloned, it is placed into a construct that is used for transformation into plants. The gene, or allele, or truncated, substituted or deleted variants thereof, can be placed in the sense or anti-sense position in a transformation vector. One of ordinary

skill will appreciate that downregulation of native genes through anti-sense does not require insertion of the entire gene in the anti-sense direction but rather only so much of the sequence as will provide down-regulation. That is, fragments of the gene, such as containing 30-100, preferably 45-65, nucleic acid bases, may be useful in down-regulating gene expression.

Transformation vectors for plant transformation can be purchased from Clontech and other commercial sources. Vectors are preferably selected to provide appropriate material for the plant species and the transformation method that is being employed. For example, if the plant species is amenable to Agrobacterium transformation then the selected vector should include the T-DNA portions in the vector. Promoters, introns, leader sequences, and selectable markers should be selected to give the desired levels of expression of the amino acids encoded by the gene in the desired plant. Moreover, vectors may be optimized by specific codon usage optimization for a given host cell which is being transformed.

Recently, inducible promoters have been used to activate genes operably linked o the promoters. The inducible promoter activates or deactivates the gene in response to an activator.

For purposes of the present invention a promoter may be either turned on and the gene or operably linked nucleic acid sequence is expressed (to include transcription alone or transcription and translation), or the promoter may be turned off and thus the gene or operably linked nucleic acid sequence is not expressed (to include transcription alone or transcription and translation). A promoter that when contacted with the activator changes the expression level can also be used. Such promoters are well known in the art and are termed "switchable" or "inducible". Inducible promoters activate the genes in response to an activator such as heat, light, moisture, chemicals and the like. U.S. Patent No. 5,608,143 describes the use of nucleic acid promoters that are highly responsive to a number of substituted benzenesulfonamides as activators. The inducible promoters are indicated for use within a recombinant DNA construct which will allow the expression of a gene to be controlled by an external chemical control agent which acts as

an activator .

U.S. Patent No. 5,432,068 describes externally inducible promoters which are used in the control of male fertility. WO 90/08826 describes a gene promoter sequence (GSTII (glutathione-S-transferase isoform II)) which responds to a plant herbicide safener (N,N-diallyl-2,2-dichloroacetamide) which was used as a gene switch to enable the external control of the gene expression. There are other external chemically inducible promoters known in the art.

Methods of transforming monocots and dicots are known. Constructs that are adapted for the plant species that is to be transformed are readily purchased or made by those having ordinary skill in the art. These constructs are then transformed into the cells or tissue or pollen of the plants. Transformation methods include but are not limited to micro particle bombardment, whiskering, electroporation, Agrobacterium, and the like. The efficiency of these techniques vary and a person of ordinary skill in the art may select the method according to the tissue type and the plant species that is to be transformed.

Selective breeding for desirable traits, such as have produced the commercially available Top Cross® line of high oil corn, is known. Once a transformant is regenerated (if necessary) it can be employed in breeding methods with other plants of its species. One of ordinary skill will appreciate that breeding practices are dependent on the species selected. For example, soybeans are developed as cultivars whereas corn is produced as a hybrid. One method of producing maize that is patented by Dupont does not use hybrid seed but a male sterile hybrid seed in combination with a male pollinator. The farmer plants not hybrid seed, but male-sterile hybrid seed and a male pollinator (maize inbred seed) that is carrying a high oil trait. Hybrid canola is being developed. Attempts are being made to move most crops that are not presently in hybrid production toward hybrid seed instead of cultivars to gain hybrid vigor but more importantly to reduce the germplasm risk associated with the production of a plant that can reproduce itself from the seed (hybrid seed segregates, inbred seed does not).

There remains a need for a grain that has added nutritional value due to reduction of the phytic acid produced in the plant. There also remains a need for a method of making seed lethal to prevent volunteer plants developing. There remains a need for an inducible lethal trait in seed of a number of plant species.

Summary of the Invention

It is an object of the present invention to provide seed, specifically maize seed, with very low phytate content.

It is another object of the present invention to provide grain, preferably nonmaize grain, that have added nutritional value due to reduction of the phytic acid produced in the plant.

It is yet another object of the invention to provide a nongerminable seed which contains at least one genetic construct which is under the control of and operably linked to a non-natural inducible promoter or other genetic element wherein the at least one genetic construct, when induced, produces a germinable seed. The seed of the present invention contains a nongerminable trait or phenotype as well as a genetic element which is under the control of an inducible promoter such that when the promoter is induced, the non-germinable trait is corrected or overcome and the seed will germinate

It is another object of the invention to provide a germinable seed which contains at least one genetic construct which is under the control of and operably linked to a non-natural inducible promoter or other genetic element wherein the at least one genetic construct, when induced, produces a nongerminable seed. The seed of the present invention contains a germinable trait or phenotype as well as a genetic element which is under the control of an inducible promoter such that when the promoter is induced, the germinable trait is destroyed or overcome and the seed will not germinate.

A further object of the present invention is to provide nucleic acid molecules (sequences) which code for proteins involved in the synthesis of phytate as well as the phytate encoded for and antibodies to same.

An object of the present invention is to provide vectors (expression and cloning), and methods for using same, for making the seed of the present invention which preferably contain the phytate encoding nucleic acid sequences, or allele, or truncated, substituted, or inserted variants or fragments thereof.

A further object of the present invention includes providing methods of producing inducible nongerminable seed and inducible germinable seeds..

Yet a further object of the present invention is a method of preventing volunteer plants from developing from fallen seed which includes planting and growing the germinateable mature seeds of the present invention and applying an activator to the plants produced therefrom, to produce daughter seeds or embryos which will not germinate.

Still further objects and advantages will become apparent from a consideration of the present description and accompanying drawings.

Brief Description of the Drawings

FIG. 1 shows a binary representation of a construct according to the present invention developed for use in dicots and those monocots capable of Agrobacteruim transformation, the construct contains the right T- DNA border, the NOS promoter, the NPTII marker gene and the NOS terminator, the inducible promoter, in this instance the GSTII promoter sequence, and the phytate gene in the sense orientation and the NOS terminator and the left T-DNA border.

FIG. 2 shows a binary representation of a construct according to the present invention developed for use in dicots and those monocots capable of Agrobacteruim transformation. The construct contains the right T- DNA border the Nos promoter the NPTII marker gene and the NOS terminator, the inducible promoter in this instance promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 and the sequence shown in Figure 5 therein (this patent incorporated by reference herein), deposited with the American Type Culture Collection (ATCC),

Manassas VA , Accession No. 67804, and the phytate gene in the antisense orientation and the NOS terminator and the left T-DNA border.

FIG. 3 shows a construct with the promoter. In this instance the promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 and the sequence shown in Figure 5 therein deposited with the American type Culture (ATCC), Manassas, VA, Accession No. 67804, the phytate gene of the present invention and the NOS terminator. The phytate gene is in the sense orientation.

FIG. 4 shows a construct with the GSTII promoter sequence, the phytate gene of the present invention and the NOS terminator. The phytate gene is in the anti-sense orientation.

FIG. 5 shows the prior art sequence of the nucleotide sequence of the 5-2 gene promoter from the gene designated 52.411 in U.S. Patent No. . 5,608,143.

FIG. 6 shows an example of data obtained from 3 inbred lines screened for phytate content, plotted as a frequency distribution curve.

FIG. 7 shows data taken from a low-phytate mutant segregating for low phytate (high-phosphorus) content.

FIG. 8 shows the nucleic and amino acid sequences of myo-inositol 1-phosphate synthase, Accession AF056326.

FIG. 9 shows a linear map of the published sequence of Zea Myo-inositol 1-phosphate synthase from base 86 to base 1620

Detailed Description

EMS (Ethyl Methane Sulfonate) has been used to induce mutant phytic acid genes in maize to produce low-phytate seed. For example, a low phytic acid maize mutant, lpa1, has been produced by Raboy (U.S. Patent No. 5,689,054). In some instances seed may be sufficiently low in phytate as to prevent germination of the seed. In other cases low phytate seed can be found that germinate normally.

From these mutated plants the gene of the present invention is isolated

and cloned in a repeatable manner. The cloned gene encodes for an enzyme that affects the production of phytic acid in the seed. The gene of the present invention includes allelic, truncated, deleted and substituted variants of the cloned gene as well as useful fragments, such as those which may be used to down-regulate the gene in an anti-sense manner, as described herein. The present invention provides a transformation vector containing the new gene operably linked to an inducible promoter so that the lethal effects of the gene can be managed, exploited or controlled.

This vector is useful in crops which are not hybrids such as soybeans, wheat, barley, maize, canola and sunflowers because the vector can be used to make seed with a very low phytic acid content and also the seed can only germinate in highly controlled conditions following induction. This vector of the present invention, when present in a plant, has the following several advantages: (i) it is useful to farmers because it eliminates volunteer plants in the next season by rendering the seed incapable of germinating, (ii) it is useful to a seed company because it maintains germplasm security by rendering the seed incapable of being reproduced for breeding purposes, (iii) it prevents farmer saved seed by rendering the seed incapable of being reproduced for future years, and furthermore, (iv) it is useful in the feed and milling industry because it adds nutritional value to the feed or milled products by being low in phytic acid content. This invention is particularly useful in the flour grains and the feed grains. These include, but are not limited to corn, wheat, soybeans, sunflower, oats, rye and the like.

The present invention provides, therefore, methods of seed security which includes providing a low-phytate, non-germinating daughter seeds or embryos which have been altered to include a functional phytate coding gene which is operably linked to, and under the control of, an inducible promoter.

Targeting and isolating gene

Genes of the present invention may be isolated, for example, by crossing with a mutant maize corn plant that has a genetic mutation that decreases the levels of phytic acid in the seed. From this gene, the wild-type

or native gene, and the mutant gene, can be identified. Both the native phytic acid gene or the mutated phytic acid gene can be employed in the present invention.

In one embodiment, the present invention provides methods to make and use seed, as well as seed and plants therefrom, wherein the phytic acid content of the seed is eliminated or severely reduced by insertion of an antisense construct of genes or fragments thereof which encode, in the sense direction, proteins involved in the synthesis of phytic acid which, even when operably linked to a weak promoter, are expected to at least severely reduce the phytic acid levels of the seed.

In another embodiment, the present invention provides methods to make and use seed, as well as plants therefrom, wherein the phytic acid content of a low-phytic acid mutant containing seed is restored by insertion of a sense construct of the native gene or fragments thereof, i.e., native gene which has been mutated to produce the low-phytic acid phenotype, which, even when operably linked to a weak promoter, are expected or known to increase the phytic acid levels of the seed.

One method of isolating the phytate encoding nucleic acid sequence of the present invention is through the use of transposon-tagged mutants which allow for cloning of the gene, by means known in the art. The transposable element tags the gene, in other words the sequences flanking the transposon insertion site are part of the gene of interest. According to the present invention, which is exemplified herein as maize, a low-phytic acid mutant inbred line is crossed to a line containing the mu1 mutator gene and a low phytic acid mutant gene is identified by the mu1 element insertion into the gene of interest. The mu1 used in the exemplified embodiments of the present invention is a part of the mu1-9 family sharing the ~20-bp terminal inverted repeat structure. Multiple copies of the transposon are contained in the mutator lines, which are aggressive mutagens in maize. Most mu insertion events give a null phenotype because the transposable element insertion does not result in altered essential proteins. With targeted transposon tagging, the insertion event is targeted to specific known

mutations, such as the low phytic acid gene. Transposon events occur in the range of ~10^{-4} to 10^{-6} insertions per locus per chromosome tested. To identify when the phytic acid gene has a DNA transposon inserted therein requires that there is identification of a mutant phenotype from the insertion event and restoration of partial or full function in the gametes from the excision events. The tagged mutant can then be crossed with a low phytic acid mutant plant carrying the recessive low phytic acid gene. If the transposable element has landed or inserted in the gene of interest then the grain from the cross can be identified as low phytic acid grain or low phytate grain. The gene is recessive and its effects are only present if the parents both carry the low phytic acid gene. The allelism is checked in a similar breeding crossing manner with similar known low phytic acid mutants.

To find the transposable event requires several hundred thousand progeny if the less active elements are used. Use of mu1 substantially reduces the required number of progeny that need to be screened for the grain trait. Depending on the transposable element used it maybe useful to backcross the inbred having the targeted mutant gene. Backcrossing was used in the present exemplification of the invention described herein because the mu1 line was used. The mu1 line can be identified as having lost mutator activity through the backcrossing procedure by monitoring the reporter gene of the anthocyanin pathway which produces a purple coloring in the seed. The backcrossing to the standard maize inbred simplifies the segregation analysis because the copy number will be greatly reduced. The allelism is checked with similar known mutants. The mu1 element is cloned using the transposon element as a probe to clone the mutant alleles or alternatively to probe for the native wildtype gene. This method identifies the low phytic acid native and mutant genes in seed, which may then be isolated, purified and sequenced, by means known in the art.

The method for finding mutable alleles, as an efficient means for cloning functional genes, requires a mutant tagged with an insertion element, a clone of the element, assays for verifying the clone recovered, and simple genetics tests. The loss of reporter gene activity is used to show loss of

mutator activity. In general, the process involves a process termed co-segregation. This involves selecting and harvesting tissue for the molecular cloning; preparing DNA from the progeny of crosses of diverse lines; and making samples from several m/m (homozygous recessive mutant genes) and several M/M (homozygous wild-type genes) individuals from each line. Next, southern blots are prepared from DNA taken from the mu-tagged material after digesting the samples with several 5-methyl cytosine-insensitive enzymes that do not digest the mu1 element that is tagging the mutant; bands are located and identified which are present in all m/m individuals that are not present in the M/M individuals; and a DNA pool of the m/m individuals are prepared and three samples from each diverse line are sampled. The darkest band as well as the only shared, diploid copy fragment are searched for and selected. This process of co-segregation will identify the mu-element which is tagging the mutant-gene. Results were verified by matching the fragment with the segregation analysis. The fragment is identified from the southern blots by locating the line with the fewest number of segregating bands that has no evidence of a co-migrating band. A large sample of m/m and M/M progeny of this line are analyzed to evaluate the statistical significance between the band and the phenotype. Various restriction enzymes are used for this confirmation. Significant samples in this population test are subjected to digestion by several enzymes, in order to screen for enzymes that do not digest the bands. This is done as a double digestion with the initial restriction enzyme as well as enzymes which poorly digest the bands, in combination with high resolution agarose gels. Then the m/m digest pool of DNA which was not digested is multiplied and the DNA is size selected and cloned.

Cloned Gene

Genes that are cloned by the process described above are genes which encode for amino acids that produce proteins which function to alter levels of phytic acid in the plant. These genes are designated as phytate genes (phytic acid genes).

Inducible promoter

A number of compounds have use in the control of gene expression in plants. The inducer of gene expression must be safe and not have detrimental effects on the desired agronomic traits of the plant.

Natural products, including hormones, and other chemicals, are known which affect gene expression in plants. Plant growth regulators which include AAA , ethylene, absisic acid, auxin, salicylic acid and other plant hormones all affect gene expression. These natural chemicals appear to induce gene expression. Hormones, whether natural or synthetic, which affect the gene expression in plants, can be used with the present invention. However, care must be taken to avoid undesired activation of the growth of the plant by the co-activation of the plants internal systems or activation of lethal genes present in the plant. Clearly, use of hormones as the inducing agent must be linked to a hormone that is not going to active the metabolic system that is harmful to the end product.

Environmental inducers for regulating foreign genes include light, heat, low temperature and different gas levels in the air. These regulators can be used, individually or in combination, in accordance with the present invention. These regulators are slightly less practicable as there is not a method presently known to control very many of these environmental inducers. It is not impossible to have a cool growing season or a cloudy growing season in which these inducers may not be activated strongly or may be activated in an untimely fashion. So although these types of inducers will work with the present invention they must be selected with care.

Other plant genes have been induced by oligosaccarides which are present in wounding and pathogen infection, for example, the induction by glucan of phenylalanine ammonia lyase and chalcone synthase in soya, or the induction of a wound-like inducible inhibition gene in potato. This induction requires more effort as the plant has to be wounded to induce the foreign gene.

The inducer of choice, that is safe and has little or no effect on the

plant to which it is applied is the most useful inducer in most instances. The preferred external control of the inducer is an agent that induces the expression of the desired gene in any tissue at any time in the plant's life cycle. This requires a promoter that is activated or deactivated upon the application of the activator material such as a chemical. This regulation is accomplished by controlling this response in a number of plant species with little effect on the plant growth. The ideal activator is a chemical that is applicable with standard field equipment, in combination with something else, such as a herbicide, that is normally applied to the field to avoid repeated passes over the field . Alternatively, the material could be applied aerially by crop dusting type equipment.

At least one embodiment of the present invention uses a promoter which is known to allow (drive) the production of enzymes which protect the plant when activated by an activator, generally referred to as chemical safeners, commonly used in conjunction with herbicides. It has been determined that plants are more protected from herbicide activity when they are safened. One example of a safener is one which acts to conjugate the herbicide with glutathione. This is due to an increase in glutathione-S transferase (GST) activity increases the mRNA of the GST in the safened plants. Thus this safener treatment increases the gene product but does not negatively effect the plant.

It has been shown that corn can be safened by a wide variety of agents, including, but not limited to, naphalic anhydride, N, N-dially-2,2-dichloroacetamide or cyometrinil when sulfonylurea herbicides are employed. The rates of the metabolism of chlosulforon and metsulfuron methyl increase within hours of the application of the safener. In the present invention, it is the promoter of the GST enzyme which is used as the switchable promoter linked to the phytate gene of the present invention. Switching is then achieved by use of a switch-inducing chemical agent.

The gene-inducer useful in the present description can be any number of inducing or activating agents. Clearly the inducing agents in either US Patent No. 5,608,143 (incorporated by reference) and/or WO 90/08826,

(incorporated herein by reference) entitled, "Gene Switch" are useful. The method of using an inducing agent to switch on a gene is known in the art and can be implemented according to the teaching of the present invention. These methods are also described in WO93/09237.

The preferred promoters of the present invention are those which respond to safeners used in herbicide formulations. In nature these promoters and their associated genes are induced by the safener in the herbicide to protect the plant from the damage that the herbicide can inflict on the plant in the absence of the safener. In the present invention, these promoters and heterologous genes are preferably activated and switched on or off by the safener. The safener is preferably applied in a herbicide and a marker gene can make the plant resistant to such herbicide if it is not already resistant. Alternatively the chemical, such as the safener, can be applied separately to the plant. This may be useful if the herbicides primary application time is significantly earlier then seed development.

The present invention provides therefore, methods to make and use seed, as well as seed and plants therefrom, wherein the phytic acid content of the seed is eliminated or severely reduced by insertion of an antisense construct of genes or fragments thereof which, in the sense direction, encode proteins or fragments of proteins involved in the synthesis of phytic acid which, even when operably linked to a weak promoter, are expected to at least severely reduce the phytic acid levels of the seed; the construct being operably linked to a promoter or other genetic element which is induced by a chemical or environmental factor, preferably a safener, such that application of the chemical or environmental factor to the plants produced from the seed produce seeds with a severely reduced amount of phytic acid when compared to the original seed.

Preferably, the seed produced from the plant which has received the factor contains less than about 5% of control phytate content by weight , more preferably less than about 2% of control phytate content by weight, more preferably less than about 1% of control phytate content by weight phytic acid than the seed which produced the plant which has received the factor.

One of ordinary skill will appreciate that common means of measuring phytic acid requires destruction of the seed such that these comparison values are most commonly average values of a sampling of seed produced as described herein. Moreover, one aspect of the present invention is to provide non-germinable seed after induction of the parent plant, irrespective of the decrease in phytic acid amount as compared to the parent seed.

In another embodiment, the present invention provides methods to make and use seed, as well as plants therefrom, wherein the phytic acid content of a low-phytic acid mutant containing seed is restored by insertion of a sense construct of the native gene or fragments thereof, i.e., native gene which has been mutated to produce the low-phytic acid phenotype of the low-phytic acid mutant, which, even when operably linked to a weak promoter, are expected or known to increase the phytic acid levels of the seed; the construct being operably linked to a promoter or other genetic element which is induced by a chemical or environmental factor, preferably a safener, such that application of the chemical or environmental factor to the plants produced from the seed produce daughter seeds with an increased amount of phytic acid when compared to the original seed. Preferably, the seed produced from the plant which has received the factor contains more than about 5% of control phytate content by weight, more preferably more than about 8% by weight, more preferably more than about 10% by weight phytic acid than the seed which produced the plant which has received the factor.

One of ordinary skill in the art will appreciate that the low or reduced phytic acid plants and seed of the present invention may be produced by transformation with nucleic acid molecules encoding phytase, preferably operably linked to an inducible promoter of the present invention, either in conjunction with the phytate genes and variants described herein, or alone. Expression of any of these phytases (enzymes of phytic acid degradation) during grain development may be used to degrade phytic acid being synthesized in the developing seed. Phytase encoding sequence are known, such as have been published by the NCBI, and elsewhere, under any of the following accession numbers: P34752 ,

gi|464382|sp|P34752|PHYA_ASPNG [464382]; P34753 , gi|464381|sp|P34753|PHYA_ASPAW [464381]; 2599490 (AF029053) phyb13 precursor [Bacillus subtilis] gi|2599490 [2599490] ; 1IHP gi|2981783|pdb|1IHP| [2981783]; 3150040 (U85968) gi|3150040 [3150040]; 2943981 (U92439) phytase [Enterobacter cloacae] gi|2943981 [2943981]; P34754 gi|464385|sp|P34754|PHYB_ASPNG [464385]; P34755 gi|464384|sp|P34755|PHYB_ASPAW [464384]; P07102 gi|130735|sp|P07102|PPA_ECOLI [130735]; 2108356 (U59802) phytase [Talaromyces thermophilus] gi|2108356 [2108356]; 2108354 (U59804) phytase [Aspergillus fumigatus] gi|2108354 [2108354]; 2108352 (U59803) phytase [Emericella nidulans] gi|2108352 [2108352]; 2300890 (A46793) gi|2300890|gnl|PID|e306401 [2300890]; 2300889 (A46793) gi|2300889|gnl|PID|e306184 [2300889]; 2300887 (A46791) gi|2300887|gnl|PID|e306400 [2300887]; 2300885 (A46789) gi|2300885|gnl|PID|e306183 [2300885];2300883 (A46787) gi|2300883|gnl|PID|e306182 [2300883]; 2300881 (A46785) gi|2300881|gnl|PID|e306399 [2300881];2300879 (A46783) gi|2300879|gnl|PID|e306181 [2300879]; 2148991 (U60412) phytase [Aspergillus terreus] gi|2148991 [2148991]; JN0715 3-phytase (EC 3.1.3.8) B precursor - Aspergillus ficuum gi|542378|pir||JN0715 [542378]; JN0890 acid phosphatase (EC 3.1.3.2) precursor - Aspergillus awamori gi|542377|pir||JN0890 [542377]; JN0889 3-phytase (EC 3.1.3.8) A precursor - Aspergillus awamori gi|542376|pir||JN0889 [542376]; JN0656 3-phytase (EC 3.1.3.8) A precursor - Aspergillus niger gi|484414|pir||JN0656 [484414]; JN0482 3-phytase (EC 3.1.3.8) A - Aspergillus ficuum gi|419906|pir||JN0482 [419906]; PQ0641 3-phytase (EC 3.1.3.8) - Aspergillus ficuum (fragments) gi|542374|pir||PQ0641 [542374]; S33278 phytase P2 - Escherichia coli (fragment) gi|421153|pir||S33278 [421153]; B36733 acid phosphatase (EC 3.1.3.2) precursor - Escherichia coli gi|96267|pir||B36733 [96267]; S18408 alkaline phosphatase (EC 3.1.3.1) - rat gi|111353|pir||S18408 111353]; 1943870 (U59806) phytase [Thielavia heterothallica] gi|1943870 [1943870];1943868 (U59805) phytase

[Aspergillus terreus] gi|1943868 [1943868]; 1938256 (U75531) phytase [Zea mays] gi|1938256 [1938256]; all sequences and text of US patent 5593963 , including, gi|1831488|pat|US|5593963|20 1831488]; 408990 phytase {EC 3.1.3.26} [Aspergillus ficuum, Peptide, 441 aa] [Aspergillus ficuum] gi|408990|bbs|130910 [408990]; 235916 gi|235916|bbs|58159 [235916]; 235915 gi|235915|bbs|58160 [235915]; 2393 gi|2393 [2393]; all sequences and text of US-5436156 , including gi|912286|pat|US|5436156|32 [912286]; 583196 (A19452) gi|583196 [583196];583194 (A19451) gi|583194 [583194];166521 (M94550) gi|166521 [166521]; 166519 (L02421) phytase [Aspergillus niger] gi|166519 [166519]; 166482 (L02420) acid phosphatase [Aspergillus niger] gi|166482 [166482]; 304097 (L20567) phyB Aspergillus niger] gi|304097 [304097]. Methods and constructs useful in the present invention may also be found in U.S. Patent No.s 5,770,413 and 5,593,963.

In a further embodiment, the construct and promoter or other genetic element are operably linked to a marker gene as are known in the art.

In one embodiment of the present invention, the plants and seed described in the embodiments of the invention are any one of maize, soybeans, rice, oats, sunflower, wheat, barley, forage grasses, and the like

Constructs

The present invention includes a construct that has a promoter associated with a phytic acid gene, the promoter is under external control such that when activated makes the seed so low in phytic acid that the seed is unable to germinate (ie it can not form a plant that matures). This construct can be transformed into any number of plants that are transformable. The positioning of the gene within the construct determines whether the gene is going to be up regulated, the sense orientation, or down regulated, the antisense orientation.

Figure 1 shows a binary representation of a construct according to the present invention. This construct is developed primarily for use with Agrobacterium transformation. Thus it is principally developed for use in dicots and those monocots capable of Agrobacteruim transformation. The

construct contains the right T- DNA border the Nos promoter the NPTII marker gene and the NOS terminator, the inducible promoter in this instance the GSTII promoter sequence and the phytate gene in the sense orientation and the NOS terminator and the left T-DNA border. This construct is ideal for soybeans and like cultivars. The marker gene can be selected to be a herbicide resistant gene such as glyphosate resistant gene.

Figure 2 shows a binary representation of a construct according to the present invention developed for use in dicots and those monocots capable of Agrobacteruim transformation. The construct contains the right T- DNA border the Nos promoter the NPTII marker gene and the NOS terminator, the inducible promoter in this instance the promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 the sequence shown in Figure 5 and the phytate gene in the antisense orientation and the NOS terminator and the left T-DNA border. This safener is applied to activate the promoter and thus express the product encoded by the gene by application of the herbicide containing the safener or just by safener application.

Figure 3 shows a construct with the promoter in this instance the promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 the sequence shown in Figure 5 therein this patent incorporated by reference herein, deposited with the American Type Culture Collection (ATCC), Manassas, VA, Accession No. 67804. The phytate gene of the present invention and the NOS terminator. The phytate gene is in the sense orientation. Thus when the activator is applied the promoter switches on and the gene will express the amino acids it encodes for.

Figure 4 shows a construct with the promoter in this instance the GSTII promoter sequence and the phytate gene of the present invention and the NOS terminator. The phytate gene is in the anti-sense orientation.

These constructs in their various forms can be transformed into maize, soybeans, rice, oats, sunflower, wheat, barley, forage grasses, and the like. The present invention includes constructs which have been optimized for expression in each of these plants, such as, for example, with respect to

codon usage, promoters, etc. and any other expression-enhancing process as well as optimised for producing an active enzyme or optimal antisense or cosuppression effects. Additionally, the constructs of the present invention can be transformed into plants that are already transformed or already mutants. For example this gene construct can be used to make the existing low phytic acid mutants externally regulated by application of the activator. The promoter in the construct can act to induce the gene to express or the activator can stop the expression of the gene by inducing the promoter to shut down. Likewise, the orientation of the gene in the construct may be in the sense or antisense direction, with sense orientation up regulating the gene expression and antisense orientation down regulating the gene expression. Thus the same effect can be produced by switching the promoters from inducible on to inducible off. or changing the orientation of the gene.

Transformation

Representative diagrams of vectors in accordance with the present invention are shown in the first four figures. The first two figures show a construct that is adapted for Agrobacteruim transformation. The second two figures show a plasmid for use with other transformation techniques such as micro particie bombardment. These transformation techniques are well known to those of skill in the art. Additionally, these constructs are excellent for use with the whiskers transformation system taught in U.S. Patent No. 5,302,523. and U.S. Patent No. 5,464,765 incorporated herein by reference.

EXAMPLE 1

There are four preferred ways to produce the low phytic acid seed material of the present invention. The preferred method provides the farmer a seed product that is both low in phytic acid and is non-germinable so that the seed is not capable of producing a mature plant. The preferred seed of the

present invention ends farmer-saved-seed in the case of cultivars which are not hybrids. Presently, most canola and wheat and soybeans are not sold as hybrids though both wheat and canola have ongoing research to develop hybrid capabilities. If the plant is not normally commercialized as hybrid seed but is instead seed like wheat and soybeans then the material according to the present invention is very readily produced.

Transformant Breeding with inbred material

If the promoter is induced to turn the gene on, and the gene is in the antisense orientation then application of the activator will produce seed with extremely low levels of phytic acid. Such seed would not germinate. The activator is applied preferably when the grain is filling or slightly prior thereto. The activator is preferably in the form of a safener that can be in a herbicide if the promoter is the GSTII or other safener-induced promoter. Thus, during seed increase the seed is not sprayed with the activator. This allows the production of seeds with normal phytic acid levels. However, when the seed is sold to the farmer for low phytic grain production then the activator must be applied to the plant to produce a seed with low or no phytic acid. Because of the low phytate in the seed, the activator will also make the seed unable to germinate. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and seed can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate; the mature seed containing a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which, in the sense direction encode phytic acid or fragments thereof or allelic variations thereof, the nucleic acid molecules being positioned in the construct in the antisense direction to the promoter, such that when the promoter is activated by an activator in a plant grown from the mature seed, a product developing seed or embryo is produced which will not germinate. The present invention provides the plant produced from the mature seed and the methods of reducing the

growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

If the promoter is induced to turn the gene off, and the gene is in the sense orientation, then the plant, absent the construct containing the promoter and gene, would have to contain a mutant form of the gene which produces very low levels of phytic acid in the seed such that the seed, absent the construct containing the promoter and gene. does not germinate. Application of the activator to the plant produced from the mature seed containing the construct of this embodiment will produce non-germinable developing seed or embryo which contain extremely low levels of phytic acid. The activator is preferably in the form of a safener in a herbicide if the promoter is or contains a fragment of the promoter shown in Figure 5. The activator is preferably a substituted benzenesulfonamides. Thus during seed increase, i.e., growth of the progeny of the mature seed of this embodiment of the invention for increasing the number of mature seed of this embodiment, the plant produced from the mature seed of this embodiment is not sprayed with the activator. However, when the mature seed of this embodiment is being grown to provide seed or grain for farmers or end-users, for production of low phytic products, then the activator must be applied to the plants produced from the mature seed of this embodiment of the invention to produce the low-phytic, non-germinable developing seed (embryo or child seed). This will result in the production a seed with very low phytic acid which will also make the seed non-germinable. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate wherein at least a portion of the sufficient amount of phytic acid is produced from a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which encode phytic acid, such that when the promoter is activated by an activator in a plant grown from the mature seed, a developing seed or embryo is produced by the plant which will not germinate. The mature seed

of this embodiment, absent the construct, does not produce suffiient phytic acid to germinate. The present invention provides the plant produced from the mature seed and the methods of reducing the growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

If the promoter is induced to turn the gene on, and the gene is in the sense orientation, then the plant would have to contain a mutant gene that is producing extremely low levels of phytic acid in the seed. Such seed would be unable to germinate. Application of the activator in this embodiment will restore germinability as increased levels of phytic acid will be produced. The activator is preferably in the form of a safener in a herbicide if the promoter is or contains a fragment of the promoter shown in Figure 5. The activator is preferably a substituted benzenesulfonamides. During seed increase, i.e., growth of the progeny of the mature seed of this embodiment of the invention for increasing the number of mature seed of this embodiment, the plant produced by the mature seed of this embodiment is sprayed with the activator. However, when the seed of this embodiment is being grown to produce seed or grain to the farmer or other user as low phytic containing seed or grain then the activator must not be applied. This will result in the production a seed with very low phytic acid which will also make the seed non-germinable. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate wherein at least a portion of the sufficient amount of phytic acid is produced from a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which encode phytic acid, such that when the promoter is activated by an activator in a plant grown from the mature seed, a developing seed or embryo is produced by the plant which will germinate. The mature seed of this embodiment, absent the construct, does not produce suffiient phytic acid to germinate. The present invention provides the plant produced from the

mature seed and the methods of reducing the growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

If the promoter is induced to turn the gene off, and the gene is in the antisense orientation, then the plant, absent the promoter and operably linked gene, would have to contain a normal or native gene that is capable of producing normal levels of phytic acid in the seed. Such seed, with the promoter and operably linked gene, will not germinate. Application of the activator will restore the production of phytic acid and hence the seed with the promoter and operably linked gene of this embodiment will germinate normally. The activator is in the form of a safener in a herbicide if the promoter is or contains fragments shown in Figure 5. The activator is preferably a substituted benzenesulfonamides. Thus during seed increase, as described above, the plant produced from the mature seed of this embodiment is sprayed with the activator. However, when the seed is sold to the farmer or other end user, as described above, as low phytic then the activator must not be applied. This will result in the production of a seed with low phytic acid and thus low phytate in the seed and the absence of the activator will also make the seed non-germinable. Thus the soybean or other plant species seed will not grow the following season as volunteer plants and can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate; the mature seed containing a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which, in the sense direction encode phytic acid or fragments thereof or allelic variations thereof, the nucleic acid molecules being positioned in the construct in the antisense direction to the promoter, such that when the promoter is activated by an activator in a plant grown from the mature seed, a developing seed or embryo is produced which will germinate. The mature seed of the present embodiment, absent the construct, is capable of making at least enough phytic acid to germinate. The antisense arrangement o the nucleic acid

molecles in the construct reduce the amount of phytic acid to the extent that the developing seed or embryos from a plant produced from the mature seed will not germinate unless activator is applied to the plant. The present invention provides the plant produced from the mature seed and the methods of reducing the growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

Other combinations of gene orientation and promoter inducibility and other activators are known. The safener or a pesticide activator is preferred as it decreases the time and expense of covering the field more then once. In other words it provides the dual utility of acting as a herbicide or pesticide and inducer to the gene(s).

Transformant Breeding with hybrid material

The number of gene orientation and mutant and wildtype combinations useful in the present invention increases when the plants are hybrid plants. A method of hybrid transformant breeding employs hybrids formed with two mutant low phytic acid inbreds, such as are described above, at least one of the mutants carrying a genetic construct which contains a transforming gene, or allelic, substituted, deleted or truncated variation thereof which was capable of encoding amino acid sequences necessary to produce phytic acid, preferably the construct contains the gene in the sense direction encoding for the wildtype phytic acid. During production, the lethal mutant carrying the transformed wildtype gene is exposed to the gene inducing spray during the period of seed production. This induces the promoter and the sense gene is turned on and the gene produces the necessary phytic acid to maintain the germination ability of the seed. This acts as the protector of the line and also as the maintainer of the line. Preferably, the producer of the seed applies the activator as opposed to the end-user, or farmer. The end-user or farmer need only plant the mature seed. When the inducible promoter is activated by a safener, as in the preferred embodiments, one of skill will appreciate that a number of plant herbicide may carry the activator that will induce the gene and care must be taken to limit or discourage the use of herbicides that

include the safener during the end-user or farmers production of the hybrid seed. Alternatively, herbicides could be formulated which do not contain the activator of the present invention to be applied separately from the activator/saffener. In this case, it is preferable that the herbicide and activator be applied separately.

Another method of the present invention uses a wildtype plant transformed with a wildtype gene, that, when positioned in a sense direction, encodes for normal levels of phytic acid. In this embodiment of the present invention, however, the wildtype gene is in the antisense direction and operably linked to an inducible promoter, as described herein. The gene is positioned in the antisense position to knock out expression of the wildtype gene in the plant when the plant is exposed to the activator chemical. This antisense gene is induced in the field by application of inducing chemical or environmental factor prior to production of grain or during development of the developing seed or embryo.

Alternatively, a natural mutant plant having lethal phytic acid levels may be used and is modified to include the wildtype gene in the sense orientation operably linked to an inducible promoter, as described herein, inserted such that when the promoter is induced in the plant, it over-expresses this gene and is able to produce daughter seed or embryos which will germinate. The inducible promoter is shutdown by the chemical. The chemical activator is applied, such as by spraying, on the plant by the end-user or farmer prior to the onset of senescence of the plant.
In this way, the mature seed contains the phytic acid genes needed to produce phytate levels needed during germination, but the gene is turned off and the chemical is not applied and the wildtype gene is not expressed.

The present invention provides therefore, a gene switch operably linked to nucleic acid molecules encoding amino acid sequences necessary to produce phytic acid. The direction of the gene within the construct and the specific promoter switch may be selected according to tne present invention by one of ordinary skill depending on the genetic and phenotypic background of the tissue to be transformed as well as the desired product (daughter seed

or embryo) to be produced.

EXAMPLE 2

The method of producing low-phytate mutants useful for transposon tagging is a known method called mutagenesis. The process is outlined in the Neuffer paper Maize Genetic Newsletter 45:146. Mutations were induced in the inbred line by treating pollen with ethyl methane sulfonate in paraffin oil according to the procedure described by Neuffer (1974). This treatment was performed on a number of inbreds from the various plant genotypes of cereal. This example will focus on the development of maize low-phytate mutants by this process. This mutagenesis process has been used to make a number of cereal mutants.

The general steps of the process of the present invention include treating inbred pollen (in this case maize) with ethyl methane sulfonate hereinafter "EMS". Inbred pollen is placed in EMS in oil for 45 minutes. A paint brush is used and the pollen is brushed on to the silks of a receptive corn ear. This forms the Mutant-1(M1) seed. Such seed are grown and self-pollinated to produce the Mutant-2 (M2) kernels. The resulting M2 kernels are tested for the low phytate phenotype.

A preferred maize mutant of the present invention which may be used as the basis for transposon tagging and phytic acid gene isolation is the Maize (Zea Mays) inbred line EX1965PY deposited in the ATCC (American Type Culture Collection, Manassas, VA) under conditions of the Budapest Treaty, on July 7, 1998, Designation No. 203034. Alternatively, *Zea maize* seeds of lpa1 (lpa1-1) and lpa2 (lpa2-1) mutant homozygotes described in U.S. Patent No. 5,689,054, which were deposited on August 15, 1996, under the terms of the Budapest Treaty at the American Type Culture Collection (ATCC) and have been assigned Accession No.s ATCC 97678 and ATCC 97679, respectively, may also be used. Preferred phytic acid genes of the present invention are those obtainable from these deposited materials, preferably by transposon tagging methods known in the art. Further preferred

nucleic acid sequences of the present invention include SEQ ID NO:1, a sequence encoding the protein shown in SEQ ID NO:2, and Figures 8 and 9, and sequences which have been published by NCBI (National Center for Biological Information) under Accession No.s g3108052 and g3108053. Nucleic acid sequences which encode the amino acid sequences of SEQ ID NO:2 and those shown in Figures 8 and 9, are also included as are allelic, substituted, deleted or truncated variations thereof which was capable of encoding amino acid sequences necessary to produce phytic acid.

EXAMPLE 3

The HVPE method is a common test for low phytate mutants. (Raboy, Maydica 35:383 (1990)). The method relies on differential migration of phosphorus compounds. After electrophoretically fractionating the compounds, a chromatogram allows a semi-quantitative assessment of the phytic acid relative to other compounds. An alternative method involves screening for higher levels of inorganic P in the grain. For example grain samples can be ground (to pass a 2 mm screen in A Wiley mill) followed by addition of either 50 mg of grain germ or 1 gram of endosperm in 15ml of 0.4 M HCL in 0.7 Na_2 SO_4. Phytic acid precipitates as an iron salt. Phosphorus in the ferric phytate precipitates and total P are determined. Phytic acid P (mg) are converted to phytic acid by a conversion factor 3.5.

The principles of phytate measurement are known. In the method employed in the present study, a solution of 5-sulfosalicylic acid and $FeCl_3$ (Wade reagent) forms a pink chromophore. Phytic acid binds iron in this solution decreasing the level of the pink color. The measurement of this loss of color can be used as an indication of phytic acid levels. Since the blank contains no phytate, all readings of samples that do contain phytate will be negative numbers. If there is too much phytate, however, the iron-phytate complex can precipitate as a milky white substance. In this case the pink color will not be present but the milky white matter will absorb light and result in falsely high readings. Thus some visual observation may be necessary.

This may necessitate using a smaller aliquot (less than 25 microliters) of the corn extract if the corn variety has high levels of phytate.

A rapid screening procedure, such as described as follows, may be used to score for putative low phytate seeds. In this procedure, a single edge razor is used to cut the kernel tip cap off just behind the black layer. The cut should transect the scutellum at a point at or near the radicle tip. Usually, 8 representative kernels were selected from each ear. The kernels are then placed, cut surface up, on a microplate that has the surface covered with cellophane tape (sticky side up). The staining procedure was completed after dissection of at least 100 families.

Staining was done with the use of a repeating pipette to place a 10 microliter drop of Wade reagent (as described below) on the cut surface of each kernel. After a few minutes the color disappeared as the phytic acid from the scutellum binds the iron in the Wade reagent. Observations were made for families that segregate for slower disappearance of the pink color relative to the others (perhaps 5 % of the total). These slower families were re-analyzed by the quantitative procedure described herein.

Phytate was quantified as follows. Individual kernels (7 to 10 from each family) were crushed in steel plate of a Carver hand-pump press (best results obtained when wells of plate lined with glycine paper and crushed with about 5000 lbs. pressure) and placed into 1.5 ml microcentrifuge tubes. 1 ml of 0.65 N hydrochloric acid was added and allowed to stand overnight. The combination was mixed by inversion the next day and allowed to settle for 5 minutes. A 15 microliter aliquot of the supernate was added to a microfuge tube with 100 microliters of buffer A (as described herein) and mixed. Low phytate mutants turn a very blue color due to the high phosphorus levels of the seeds. Mutants were generally retested the following day. The 0.65 N HCl extraction solution was made by adding 216 ml of 12.1N HCl to 3784 ml water. Reagent A was made fresh daily and included 2 parts (by volume) deionized water, 1 part (by volume) ascorbic acid solution, 1 part (by volume) ammonium molybdate solution and 1 part (by volume) H_2SO_4 solution. Ammonium molybdate solution was made by adding 25 g $(NH_4)_6 MO_7O_{24}$ x

$4H_20$ to make 1L with water. H_2SO_4 solution was made by adding 167 ml of 36 N sulfuric acid to 833 ml water. Ascorbic acid solution was made by adding 100 g L-ascorbic acid to make 1L with water. Ascorbic acid solution was stable with refrigeration for about seven weeks but only about two hours unrefrigerated.

Phytic Acid standards were prepared by means known in the art.

Maize kernel (M2) free phosphate was visually screened as follows. Kernels were selected, the phenotypes noted and placed in a multiple well crushing plate. Kernels were crushed in the multiple well crushing plate using a hydraulic press. Crushed kernels were transferred into 1.5 ml eppendorf microcentrifuge tubes. 0.5 ml of Reagent A was added. After allowing 2 hours reaction time, 0.5 ml reagent B was added. The tubes were capped and mixed by inverting. The reactions were scored visually for blueness after 1 hour, using a light box where necessary, and the bluest samples were selected as having highest phosphate. Often, the bluest samples from each family (ear) were selected and compared for final selection. Reagent A of this assay was prepared from 50 ml DMSO and 50 ml Reagent B. Reagent B was made fresh daily and prepared from 60 ml distilled water; 30 ml of 10% ascorbic solution (10 g ascorbic acid water to 100 ml total volume; the ascorbic solution was refrigerated and stable for 1 week); 30 ml of 3.5% ammonium molybdate solution (2.5 g $(NH4)_6\ Mo_7\ 0_{24}{}^*4H_20$ add water to 100 ml total volume); 30 ml of 6N sulfuric acid solution (170 ml water plus 25 ml concentrated H_2SO_4, adjust to 200 ml total volume with water).

Phytate (Red Test) was quantitatively measured as follows

Approximately 12 mature seeds were crushed in a steel crush plate of a Carver hand-pump press. Best results were obtained when wells of the plate were lined with weighing paper. Kernels were crushed with 5000 to 10000 lbs. pressure and transferred to Eppendorf tubes. 1 ml of 0.65 N HCl was added to same, allowed to sit overnight and mixed the following day by tube inversion. To assay, 200 ml of Wade-A reagent (described below) was combined with 10 ml of the above obtained corn/HCl juice extract in individual wells of a microtiter plate. Any change in color was noted and samples which

remained red were noted as low in phytate since phytate binds with iron and turns the solution white. Quantitation may be completed with a spectrophotometer measuring at 490 nm. Wade-A reagent used herein was prepared by adding 25.4 g of 5-sulfosalicylic acid and 350 mg of $FeCl_3.6H_2O$ (ground with mortar and pestel if necessary) to 1.5 L deionized water. NaOH was used to adjust the pH to 3.05 and volume adjusted with $d.H_20$ to 2L. This reagent was stable in a refrigerator for about 1 mo. 0.65N HCl was prepared by adding 216 ml HCl (12.IN) to 3784 .:l of $d.H_2O$.

These assays allow the selection of the desired maize plants containing the desired alleles. Further details of these procedures and embodiments according to the present invention are found in the copending PCT Application No. (Unassigned), filed July 7, 1998 entitled "Animal Feed with Low Phytic Acid, Oil Burdened and Protein Laden Grain", which is based on U.S. Provisional Application No.s 60/051,854 and 60/051,855, filed July 7, 1997, the entire contents of which are incorporated herein by reference. Other methods of testing for phosphorus are known and can be used to select plants.

Seed containing the desired levels of phytic acid are then increased. This process was employed in the present invention and a number of inbred lines were selected that carried the low phytic acid mutation. These included several low phytate inbred lines with good combining ability which were crossed together to form a hybrid that produced the grain of the present invention. Thus, the developed inbreds of the present invention were produced from stiff stalk, Lancaster and another versatile heterotic patterns so that the inbreds when crossed together with the appropriate heterotic pattern formed excellent hybrid material. It was also discovered that a number of the developed mutations of the present invention though low in phytic acid were not the same mutant as the lpa1-R and lpa2-R mutations described in U.S. Patent No. 5,689,054 as lpa1-1 and lpa2-1, respectively. Additionally, the seed were screened for germinability in standard seed germination tests. It was found that some low phytate mutants were unable to germinate whereas others would germinate normally. Only the seed with

good germination characteristics were maintained.

Figure 6 shows an example of some data obtained from 3 such inbred lines screened for phytate content. Plotted as a frequency distribution curve it is clearly evident that there were samples with very low phytate content (less than 0.5 units (weight percent)) whereas the bulk of the samples were higher in phytate content. The line known as UO95 line was selected as a starting material for its higher than average protein and oil levels. This line produces seed which are oil burdened and protein laden and which germinate normally. UO95py retains these characteristics with the low phytate levels described above. When crossed with certain other inbred lines, the resulting hybrid produces grain which are oil burdened and protein laden and contain low phytic levels.

The following table represents phytic acid contents (mg/g of seed) for mutant corn according to the present invention compared with wild-type seed.

No. Seeds	Wild-type Line	Phytic acid	Mutant Line	Phytic acid	% reduction
24	UU01	1.16	UU01-py	0.17	85.3
12	UO95	1.85	UO95-py	0.14	92.4
12	WD22	1.45	WD22-py	0.05	96.6

The following provides an example of an inbred line according to the present invention.

Inbred	Protein	Oil	Phytate	
Wild-type				
UO95	13.4	606	1.85	
UU01	12.7	2.9	1.16	
B73	11.3	4.4	---	
WD22	---	---	1.45	
Mutants	Protein	Oil	Phytate	% phytate reduction

UO95py	14.4	5.3	0.14	85.3
UU01py	12.2	3.1	0.17	92.4
B73lpa1-R	13.2	3.2	---	---
WD22py	---	---	0.05	96.6

Protein and oil contents were measured by NIR analysis on a Dickey-John Reflectance Near Infra Red Spectrometer.

The grain of the present invention can also be used as a substitute source for the corn grain or flour used to make corn tortilla, corn meal, and cornflakes by substituting the grain of the present invention in the recipe and baking or processing as one would normally.

The grain of the present invention can also be used as a substitute for the corn wet milling industry by substituting the grain of the present invention in order to increase milling efficiency and recoverable starch content. Animal feed made as a by-product of the milling process is also substantially reduced in phytate content.

EXAMPLE 4

Methods of selecting for low phytate have been described above. Figure 7 shows data taken from a low-phytate mutant segregating for low phytate (high-phosphorus) content. Clear evidence for Mendialian segregation is apparent.

EXAMPLE 5

By crossing low-phytate mutants with a population containing mutator (transposon-tagged) it is possible to identify a rare case of a mutator-tagged gene. First, homozygous low-phytate plants are crossed with a transposon-tagged population. Next biochemical assays are used to screen the resulting seed for low-phytate content (phosphate content in this case). Using this method it is possible to identify a transposon-tagged phytate-gene specifying

the gene for the low-phytate mutation. The results of the biochemical screens are displayed in Figure 7. Here are displayed the data for the frequency distribution of seed of transposon-tagged material assayed for phosphorus content. One rare event was found in this example.

Germination tests revealed that some low-phytate mutants had acceptable germination whereas others would not germinate. Similar results may be obtained by ordinarily skilled artisans using the methods described herein.

Event	Phytate Mutants	S98 germination
mUU01py292	Low Phytate	94%
mUO95py1656	Low Phytate	51%
mUO95py1212	Low Phytate	50%
mUO95py1672	Low Phytate	33%
mUO95py2148	Low Phytate	77%
mUO95py2236	Low Phytate	48%
mWD22py1104	Low Phytate	48%
mWD22py1857	Low Phytate	29%
mWD22py2016	Low Phytate	0%
mWD22py1511	Low Phytate	78%
mTR306py510	Low Phytate	61%
TR335-E338phy	Low Phytate	0%
TR335-E75phy	Low Phytate	15%
UE95-E3645phy	Low Phytate	90%
UE95-E3654phy	Low Phytate	53%
UE95-E3746phy	Low Phytate	0%

From these preferred low phytate, non-germinating materials, it is possible, using known methods, to clone, sequence and manipulate the phytic acid mutants useful in the present invention.

* * * * * *

The entire contents of references referred to herein are incorporated in their entirety by reference.

We claim:

1. A transgenic plant containing a genetic construct comprising a heterologous nucleic acid sequence encoding for a selected gene product that regulates said plant's production of phytic acid.

2. A transgenic plant according to claim 1 wherein said nucleic acid sequence is operably linked to a promoter that is inducible by an activator.

3. A transgenic plant according to claim 1 wherein said plant also contains a mutant allele that results in the plant forming low phytic acid levels.

4. A transgenic plant according to claim 1 wherein said plant also contains a wild-type phytate gene that results in the plant forming phytic acid levels sufficient for the plant, absent said construct, to produce daughter seed which will germinate.

5. A transgenic plant according to claim 3 wherein said promoter is active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the sense direction.

6. A transgenic plant according to claim 3 wherein said promoter is not active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the sense direction.

7. A transgenic plant according to claim 4 wherein said promoter is not active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the antisense direction.

8. A transgenic plant according to claim 4 wherein said promoter is active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the antisense direction.

9. A transgenic plant according to claim 3 wherein the nucleic acid sequence encodes a mutant phytate gene product obtainable from a maize mutant plant selected from the group consisting of UO95py, lpa1-1 (ATCC Accession No. 97678), lpa2-1 (ATCC Accession No. 97679) and allelic, truncated, substitution and deletion variants thereof.

10. A transgenic plant according to claim 9 wherein said gene product is obtainable by mu transposon tagging.

11. A transgenic plant according to 4 wherein said wild-type phytate gene is obtainable by mu transposon tagging phytic acid containing plants.

12. A transgenic plant according to claim 4 wherein said wild-type phytate gene is a nucleic acid sequence encoding SEQ ID NO:2.

13. A transgenic plant according to claim 4 wherein said wild-type phytate gene is SEQ ID NO:1.

14. A transgenic plant according to claim 1 wherein said plant is selected from the group consisting of maize, soybeans, rice, oats, sunflower, wheat, barley, rye, and forage grasses.

15. A transgenic plant according to claim 2 wherein said activator is a compound selected from the group consisting of: 2-chloro-N-(methylaminocarbonyl)benezenesulfonamide, 1-(n-butyl)-3-methylsulfonylurea, methyl 2-[(aminocarbonyl) aminosulfonyl]benzoate, N-isopropylcarbamoylbenezenesulfonamide, N-(aminocarbonyl)-2-chlorobenezenesulfamide and N'-[2-(n-butylaminocarbonyl)]-6-chloro-N,N-dimethyl-1,2-benezenedisulfonamide.

16. A recombinant genetic construct capable of transforming a plant, comprising

a nucleic acid sequence encoding for a gene product that regulates the production of phytic acid operably linked 3' to a nucleic acid promoter sequence.

17. A recombinant genetic construct according to claim 16 wherein said nucleic acid promoter sequence is as shown in figure 5.

18. A recombinant genetic construct according to claim 16 wherein said nucleic acid promoter sequence is inducible by application of N,N-diallyl-2,2-dichloroacetamide.

19. A recombinant genetic construct according to claim 16 wherein said nucleic acid promoter sequence is inducible by a compound selected from the group consisting of: N,N-diallyl-2,2-dichloroacetamide, benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate, naphtalene-1,8 dicarboxylic anhydride, 2-dichloromethyl-2-methyl-1,3-dioxolane.

20. A method of generating seeds comprising the following steps:

planting a mature seed containing an externally inducible recombinant DNA vector which can be activated by an activator, said recombinant DNA vector containing at least one nucleic acid sequence coding for a selected gene product that results in lethally low production of phytic acid;

applying the activator to the plant generated by said mature seed whereby the nucleic acid sequence is induced to produce said selected gene product;

harvesting the daughter seed produced from the plant after the activator has been applied.

21. A method of generating seeds comprising the following steps:

planting a mature seed containing an externally inducible recombinant DNA vector which can be deactivated by an activator, said recombinant DNA vector containing at least one nucleic acid sequence coding for a selected gene product that results in lethally low production of phytic acid;

applying to the plant generated by said mature seed, the activator whereby

the nucleic acid sequence is induced to stop encode said selected gene product;

harvesting the daughter seed produced from the plant after the activator has been applied.

22. Daughter seed produced by the transgenic plant according to claim 5.

23. Daughter seed produced by the transgenic plant according to claim 6.

24. Daughter seed produced by the transgenic plant according to claim 7.

25. Daughter seed produced by the transgenic plant according to claim 8.

26. A plant as claimed in claim 1 which is dicotyledonous.

27. A plant as claimed in claim 1 which is monocotyledonous.

28. A plant as claimed in claim 1 which is of the family *Gramineae*.

29. A hybrid plant of which at least one parent is a plant according to claim 1.

30. Daughter seed produced by a plant according to claim 1.

31. Non-germinating grain produced from a plant according to claim 1.

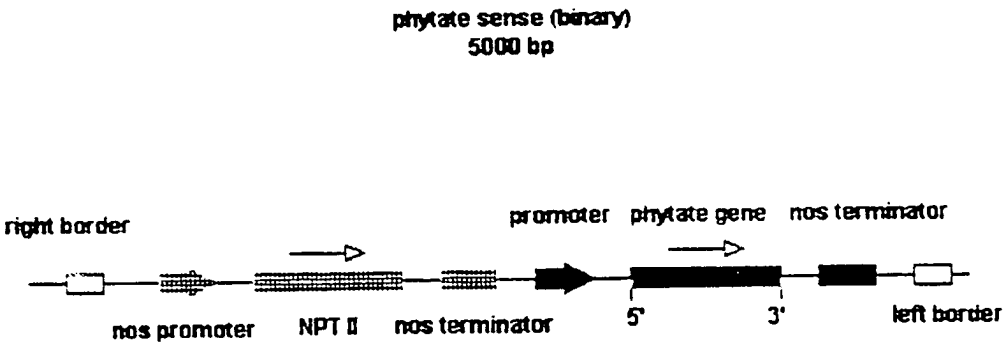
32. An isolated, purified nucleic acid sequence encoding phytate produced according to a method comprising:

crossing a low-phytate homozygous mutant plant with a population of plants containing a mutator;

identifying mutator-tagged daughter seed which contain a low-phytate content; and

cloning and isolating said nucleic acid sequence from said daughter seed.

FIGURE 1


phytate sense (binary)
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promoter
phytate gene
nos terminator
nos promoter
NPT II
nos terminator
5'
3'
left border

FIGURE 2

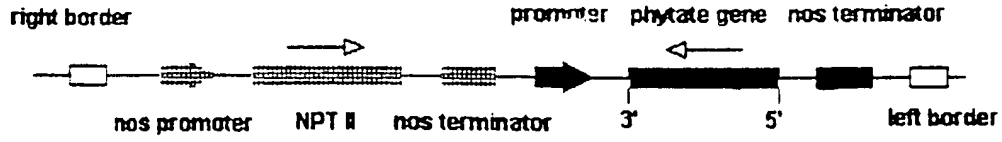
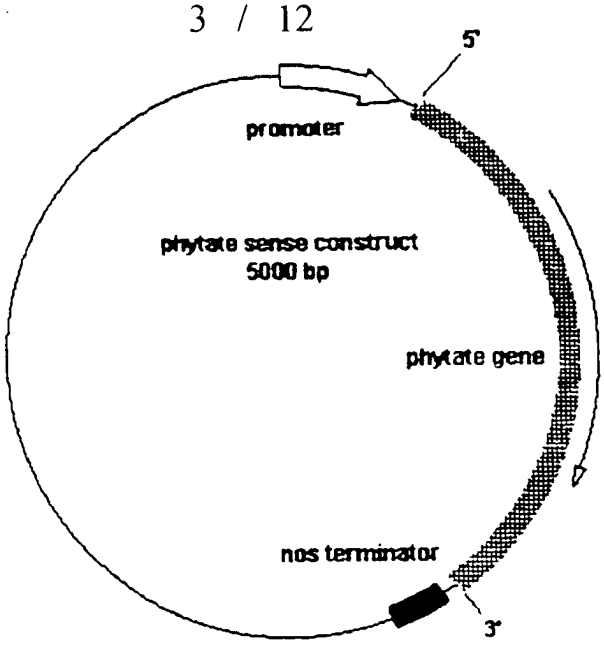

phytate antisense (binary)
5000 bp
right border
promoter
phytate gene
nos terminator
nos promoter
NPT II
nos terminator
3'
5'
left border

Fig 3


5'
promoter
phytate sense construct
5000 bp
phytate gene
nos terminator
3'

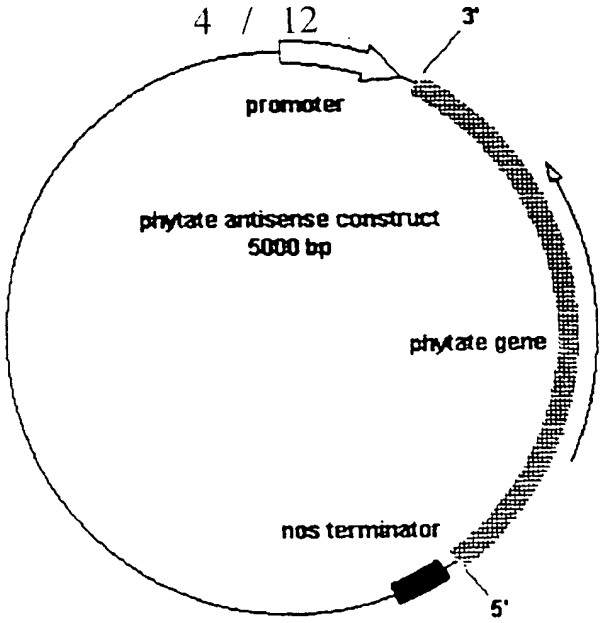

3'
promoter
phytate antisense construct
5000 bp
phytate gene
nos terminator
5'

Fig 4

FIGURE 5

(i i) MOLECULE TYPE: DNA (genomic)

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ACTGAAGAAT GATGAGTGAC TCACAAAATG GTTTCCCATT GTGGATCAAG AATGGGATTT   60
TCTTGTGAAT TGGGTTCATT TGTAGGAGCA GAGGACTTTT GATCCTCAAG TCCTCCTTCC  120
```

```
TTGTATTCAT AATGAATTCC TTTTTCAGCC AGGGCAATCC TGACCCTCAT CCCAAACATA  180
CTGTAAGTAT CTAGTAGGAC AATTTCATCT GCCTTTTTTT TTAAAATGAA ATTTAAGGAT  240
AGTATAATGG AATTCCAACA AATATAAAAC TAGAATCAGT TATTATTCAA CATAAACCCA  300
TGAAGTACCA AATTTGTGGG GGTAGAGAGA AGATTTGGAT CGACTAAAAT TTTGACTAGT  360
AAGTTAAAAA AATTAAGGAA CAGAAGAAAG TGGAGCCTTC TTGCTTAACG TTTACTACTA  420
TAAGACCCCG TGACGAATGT GATGACATAA GTAGGTCGGC CACACAAAAA AATCTGGAAA  480
CTCCCGGACC ACAACACCGC TTGTACCCAT AATAAAAATG TTTAAAAATG AAGACATCTA  540
AGTTTCTACT GGTCTATATA TAGAACTTGA ACTATATACG AAGCATATCA GTTCTAAGCA  600
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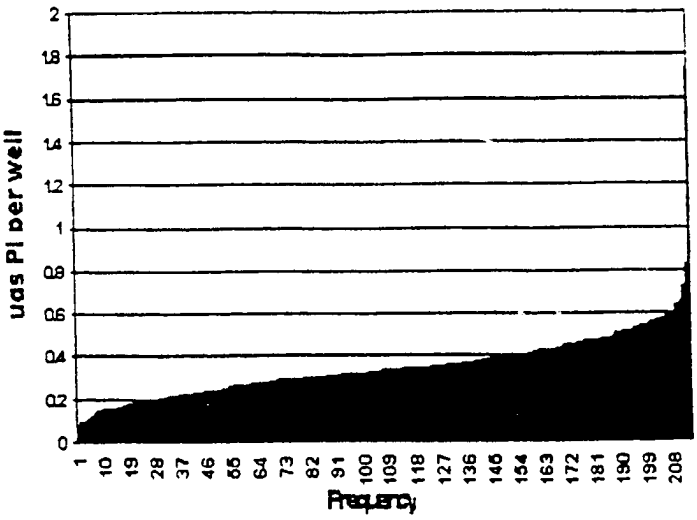
Fig 6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1.8
1.6
1.4
1.2
1
0.8
0.6
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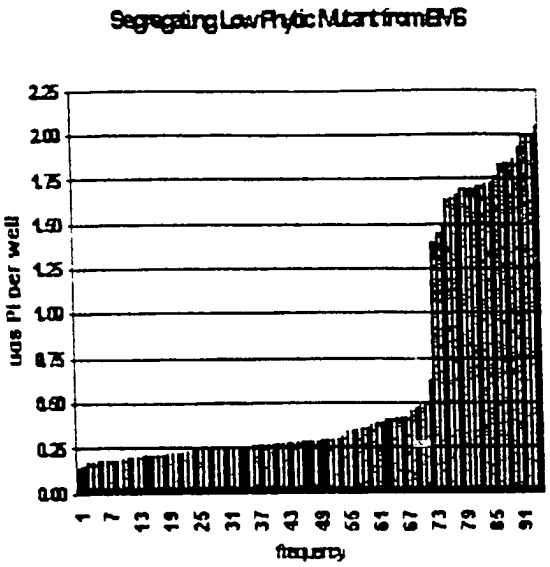
0.2
0
1
10
19
28
37
46
55
64
73
82
91
100
109
118
127
136
145
154
163
172
181
190
199
208
Frequency

FIGURE 7


Segregating Low Phytic Mutant from EMS
ugs Pi per well
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2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1
7
13
19
25
31
37
43
49
55
61
67
73
79
85
91
frequency

Figure 8 -1

```
LOCUS     AF056326    1665 bp    mRNA          PLN     03-MAY-1998
DEFINITION  Zea mays myo-inositol 1-phosphate synthase mRNA, complete cds.
ACCESSION   AF056326
NID       g3108052
KEYWORDS    .
SOURCE    Zea mays.
  ORGANISM  Zea mays
            Eukaryota; Viridiplantae; Charophyta/Embryophyta group;
            Embryophyta; Tracheophyta; seed plants; Magnoliophyta; Liliopsida;
            Poales; Poaceae; Zea.
REFERENCE   1  (bases 1 to 1665)
  AUTHORS   Larson,S.R. and Raboy,V.
  TITLE     Linkage mapping maize and barley myo-inositol 1-phosphate synthase
            genes
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1665)
  AUTHORS   Larson,S.R. and Raboy,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-MAR-1998) National Small Grains Germplasm Reserach
            Facility, USDA-ARS, 1691 South 2700 West, Aberdeen, ID 83210, USA
FEATURES             Location/Qualifiers
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                     /chromosome="1S"
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                     /tissue_type="leaf"
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                     /note="biosynthetic enzyme; INO1"
                     /codon_start=1
                     /product="myo-inositol 1-phosphate synthase"
                     /db_xref="PID:g3108053"

     /translation="MFIESFRVESPHVRYGPMEIESEYRYDTTELVHEGKDGASRWVV

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     NY
```

FIGURE 8-2

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IDLQKQLRPYMESMVPLPGIYDPDFIAANQGSRANSVIKGTKKEQVEQIIKDIREFKE

KNKVDKIVVLWTANTERYSNVCAGLNDTMENLLASVDKNEAEVSPSTLYAIACVMEG
V

PFINGSPQNTFVPGLIDLAIKNNCLIGGDDFKSGQTKMKSVLVDFLVGAGIKPTSIVS

YNHLGNNDGMNLSAPQAFRSKEISKSNVVDDMVSSNAILYEPGEHPDHVVVIKYVPYV

GDSKRAMDEYTSEIFMGGKNTIVLHNTCEDSLLAAPIILDLVLLAELSTRIQLKAEGE

DKFHSFHPVATILSYLTKAPLVPPGTPVVNALAKQRAMLENIMRACVGLAPENNMILE
YK"

BASE COUNT 405 a 461 c 478 g 321 t

ORIGIN

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121 cgtgcggtac ggcccgatgg agatcgagtc ggagtaccgg tacgacacga cggagctggt
181 acacgagggc aaggacggcg cctcacgctg ggtcgtccgc cccaagtccg tcaagtacaa
241 cttccggacc agaaccgccg tccccaagct cggggtgatg cttgtggggt ggggaggcaa
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421 agtcggcagc tacaacgggg aggagatcta tgcgccgttc aagagcctcc ttcccatggt
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541 catgaccagg gccaaggtgc tggatattga cctgcagaag cagctcaggc cctacatgga
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1261 gggagacagc aagagggcta tggacgagta cacctcagag atcttcatgg gcggcaagaa
1321 caccatcgtg ctgcacaaca cctgtgagga ctcgctcctc gccgcaccta tcatccttga
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1561 catgagggcc tgcgttgggc tggccccaga gaacaacatg atcctggagt acaagtgagc

FIGURE 9-1

(Linear) MAP of Zea Myo-inositol 1-phosphate synthase from: 86 to: 1620

```
      86 atgttcatcgagagcttccgcgtcgagagcccccacgtgcggtacggcccgatggagatc 145
         tacaagtagctctcgaaggcgcagctctcgggggtgcacgccatgccgggctacctctag

a        M  F  I  E  S  F  R  V  E  S  P  H  V  R  Y  G  P  M  E  I  -

     146 gagtcggagtaccggtacgacacgacggagctggtacacgagggcaaggacggcgcctca 205
         ctcagcctcatggccatgctgtgctgcctcgaccatgtgctcccgttcctgccgcggagt

a        E  S  E  Y  R  Y  D  T  T  E  L  V  H  E  G  K  D  G  A  S  -

     206 cgctgggtcgtccgccccaagtccgtcaagtacaacttccggaccagaaccgccgtcccc 265
         gcgacccagcaggcggggttcaggcagttcatgttgaaggcctggtcttggcggcagggg

a        R  W  V  V  R  P  K  S  V  K  Y  N  F  R  T  R  T  A  V  P  -

     266 aagctcggggtgatgcttgtgggtggggaggcaacaacgggtccacgctgacggctggg 325
         ttcgagccccactacgaacaccccacccctccgttgttgcccaggtgcgactgccgaccc

a        K  L  G  V  M  L  V  G  W  G  G  N  N  G  S  T  L  T  A  G  -

     326 gtcattgccaacagggaggggatctcatgggcgaccaaggacaaggtgcagcaagccaac 385
         cagtaacggttgtccctccccctagagtacccgctggttcctgttccacgtcgttcggttg

a        V  I  A  N  R  E  G  I  S  W  A  T  K  D  K  V  Q  Q  A  N  -

     386 tactacggctccctcacccaggcctccaccatcagagtcggcagctacaacggggaggag 445
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a        Y  Y  G  S  L  T  Q  A  S  T  I  R  V  G  S  Y  N  G  E  E  -

     446 atctatgcgccgttcaagagcctccttcccatggtgaacccagacgacattgtgttcgga 505
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.a       G  W  D  I  S  N  M  N  L  A  D  S  M  T  R  A  K  V  L  D  -

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a        I  D  L  Q  K  Q  L  R  P  Y  M  E  S  M  V  P  L  P  G  I  -

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FIGURE 9-2

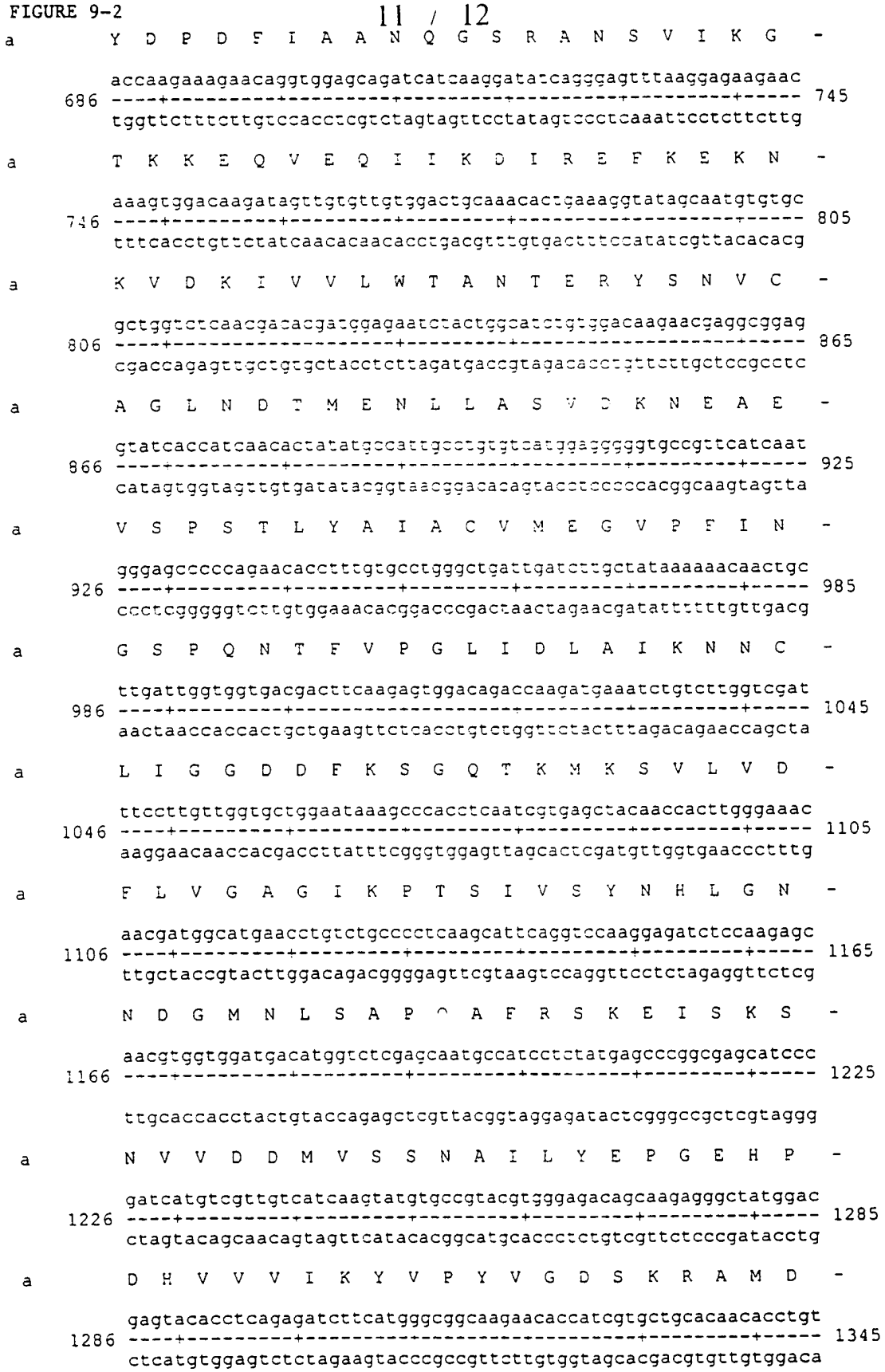
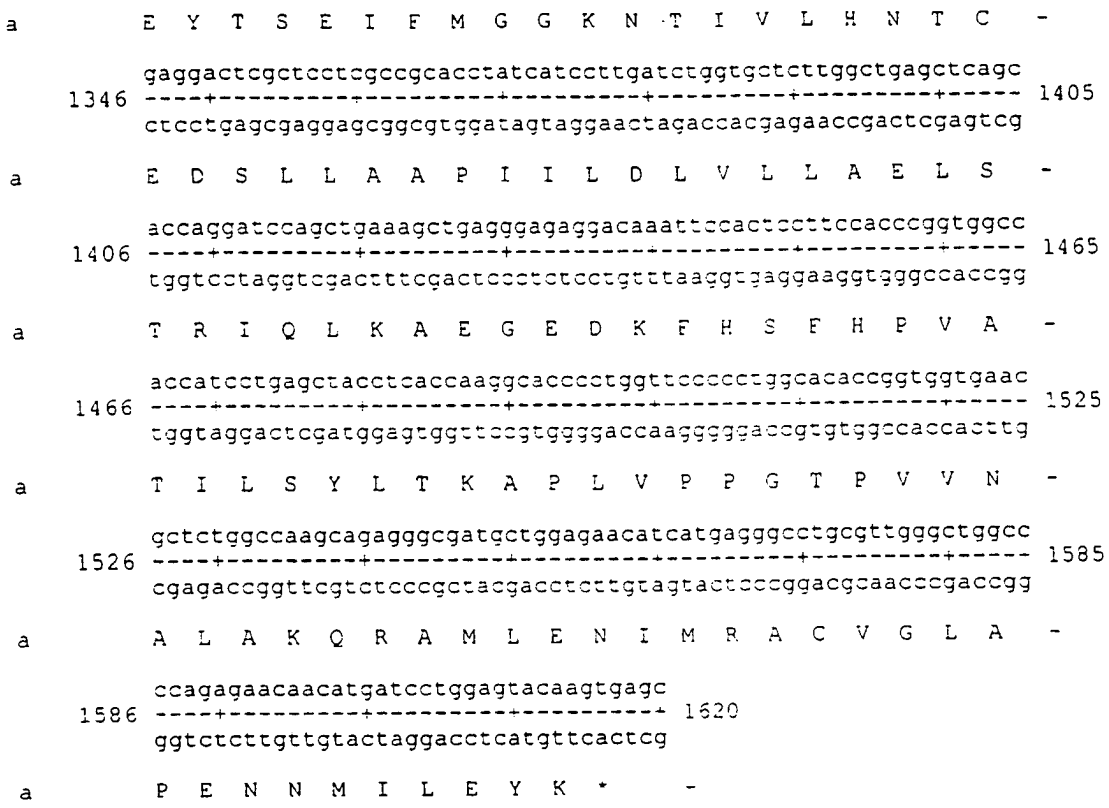


FIGURE 9-3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SEQUENCE LISTING

<110> KEELING, PETER L.
Guan, Hanping
Chang, Ming-Tang
Wilhelm, Edward P.

<120> CONTROLLED GERMINATION USING INDUCIBLE PHYTATE GENE

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16702

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 5/10; C12N 15/55; C12N 15/82; A23B 4/12

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/250; 435/068.1, 069.1, 094, 195, 200, 204, 209, 232, 233, 234; 426/007, 061, 531, 635.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS,CAPLUS,MEDLINE,AGRICOLA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	VERWOERD et al. Stable Transformation of Aspergillus Niger Phytase in Transgenic Tobacco Leaves. Plant Physiol. 1995, Vol. 109, pages 1199-1205. See the entire abstract.	1-6,16-17 --- 7-15,18-32
Y	HENNING et al. Pathogen, Salicylic Acid and Developmental Dependent Expression of a Beta-1,3-Glucanase/GUS Gene Fusion in Transgenic Tobacco Plants. The Plant Journal. 1993, Vol. 4, pages 481-493. See the entire documenation.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 OCTOBER 1998	Date of mailing of the international search report 02 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer OUSAMA M-FAIZ ZAGHMOUT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16702

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HARING et al. The Use of Transgenic Plants to Understand Transposition Mechanisms and to Develop Transposon Tagging Strategies. Plant Molecular Biology. 1991, Vol. 16, pages 449-461. See the entire documentation.	1-32

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US98/16702

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/250; 435/068.1, 069.1, 094, 195, 200, 204, 209, 232, 233, 234; 426/007, 061, 531, 635.

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CORRECTED VERSION*

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US 60/055,323 (CIP)
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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CONTROLLED GERMINATION USING INDUCIBLE PHYTATE GENE

(57) Abstract

The present invention provides a plant containing a phytic acid gene that is expressed in the seed of the plant only when desired. The present invention has two traits that can be induced or switched. The traits are (i) nongerminable seed and, (ii) low or no production of phytic acid.

*(Referred to in PCT Gazette No. 17/1999, Section II)

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Controlled Germination Using Inducible Phytate Gene

The present invention is based on, and claims benefit to U.S. Provisional Application No. 60/055,323, filed August 11, 1997, the entire contents of which is incorporated herein by reference.

Field of Invention

The present invention provides a plant containing an inducible gene (phytate gene) that controls seed germinability by regulating phytic acid content as well as methods of making and using the plant, seed therefrom, and progeny thereof

Background

Low phytic mutants in maize are known. The age of recombinant DNA has recently become a financial success in the agricultural industries. Herbicide resistant and insect resistant cotton has been successfully marketed, as have soybeans and corn. Before transformed plants there were some crops that carried herbicide resistance due to mutations in the crops. For example, IT ™ corn produced by Garst Seed Company is a mutant plant that is herbicide resistant (Imazethapyr) HERBICIDE PURSUIT® (American Cyanamid).

Mutant plants have also been used to produce added value traits in a number of crops. For example, in maize, waxy, amylose extender and other mutants are used to produce special starch traits, and, in sweet corn, the sugary mutation is utilized.

A low phytic acid maize mutant, lpa1-R, has been produced by Raboy (U.S. Patent No. 5,689,054 (lpa1-1, described therein) . The commercial introduction of this lpa1 mutant is expected to occur in 1999. A number of

companies are presently testing this new low phytic acid mutant. A number of different mutants in maize and different species which carry this low phytic trait can be produced by the use of mutating agents and mutational methods of breeding corn.

Transposon tagging is a known method of gene identification and sequencing which involves mutating plants with a transposable element. As one advantage, this method provides a means of identifying and sequencing the gene associated with the mutation. Once a gene has been cloned it can be transferred between species. Methods of moving genes from one plant species to another plant species through the use of gene identification, sequencing and transformation are well known in the industry. Annu. Rev. Plant Physiol. Plant Molecular Biology 1992. 43: 49-82 by Virginia Walbot, entitled "Strategies for Mutagenesis and Gene Cloning Using Transposon Tagging and T- DNA Insertional Mutagenesis". A specific example of using transposon tagging employing the mu1 element for the genetic isolation, and cloning of an amylose extender gene in maize was reported in the Plant Cell, Vol. 5, pp.1555-1566, (November 1993) by Philip Stinard et al, entitled "Genetic Isolation, Cloning and Analysis of a Mutator-induced, Dominant Antimorph of the Maize Amylose Extender1 Locus." This article reports mutant plants subjected to transposon tagging and the identification of the mutated gene. The gene was sequenced by methods known in the art. The sequenced portion was used as a probe to identify the mutant gene, the gene was then cloned. Cloning of genes has been performed and is well established as a technique. Techniques of DNA recombination used throughout this invention are known to those skilled in the art and are described in Maniatis et al., MOLECULAR CLONING: a Laboratory Manual, Cold Spring Harbor Laboratory , Cold Spring Harbor, N.Y.)

Switchable or inducible transformation constructs are known and may be made by means known in the art. Often, after a gene is cloned, it is placed into a construct that is used for transformation into plants. The gene, or allele, or truncated, substituted or deleted variants thereof, can be placed in the sense or anti-sense position in a transformation vector. One of ordinary

skill will appreciate that downregulation of native genes through anti-sense does not require insertion of the entire gene in the anti-sense direction but rather only so much of the sequence as will provide down-regulation. That is, fragments of the gene, such as containing 30-100, preferably 45-65, nucleic acid bases, may be useful in down-regulating gene expression.

Transformation vectors for plant transformation can be purchased from Clontech and other commercial sources. Vectors are preferably selected to provide appropriate material for the plant species and the transformation method that is being employed. For example, if the plant species is amenable to Agrobacterium transformation then the selected vector should include the T-DNA portions in the vector. Promoters, introns, leader sequences, and selectable markers should be selected to give the desired levels of expression of the amino acids encoded by the gene in the desired plant. Moreover, vectors may be optimized by specific codon usage optimization for a given host cell which is being transformed.

Recently, inducible promoters have been used to activate genes operably linked o the promoters. The inducible promoter activates or deactivates the gene in response to an activator.

For purposes of the present invention a promoter may be either turned on and the gene or operably linked nucleic acid sequence is expressed (to include transcription alone or transcription and translation), or the promoter may be turned off and thus the gene or operably linked nucleic acid sequence is not expressed (to include transcription alone or transcription and translation). A promoter that when contacted with the activator changes the expression level can also be used. Such promoters are well known in the art and are termed "switchable" or "inducible". Inducible promoters activate the genes in response to an activator such as heat, light, moisture, chemicals and the like. U.S. Patent No. 5,608,143 describes the use of nucleic acid promoters that are highly responsive to a number of substituted benzenesulfonamides as activators. The inducible promoters are indicated for use within a recombinant DNA construct which will allow the expression of a gene to be controlled by an external chemical control agent which acts as

an activator .

U.S. Patent No. 5,432,068 describes externally inducible promoters which are used in the control of male fertility. WO 90/08826 describes a gene promoter sequence (GSTII (glutathione-S-transferase isoform II)) which responds to a plant herbicide safener (N,N-diallyl-2,2-dichloroacetamide) which was used as a gene switch to enable the external control of the gene expression. There are other external chemically inducible promoters known in the art.

Methods of transforming monocots and dicots are known. Constructs that are adapted for the plant species that is to be transformed are readily purchased or made by those having ordinary skill in the art. These constructs are then transformed into the cells or tissue or pollen of the plants. Transformation methods include but are not limited to micro particle bombardment, whiskering, electroporation, Agrobacterium, and the like. The efficiency of these techniques vary and a person of ordinary skill in the art may select the method according to the tissue type and the plant species that is to be transformed.

Selective breeding for desirable traits, such as have produced the commercially available Top Cross® line of high oil corn, is known. Once a transformant is regenerated (if necessary) it can be employed in breeding methods with other plants of its species. One of ordinary skill will appreciate that breeding practices are dependent on the species selected. For example, soybeans are developed as cultivars whereas corn is produced as a hybrid. One method of producing maize that is patented by Dupont does not use hybrid seed but a male sterile hybrid seed in combination with a male pollinator. The farmer plants not hybrid seed, but male-sterile hybrid seed and a male pollinator (maize inbred seed) that is carrying a high oil trait. Hybrid canola is being developed. Attempts are being made to move most crops that are not presently in hybrid production toward hybrid seed instead of cultivars to gain hybrid vigor but more importantly to reduce the germplasm risk associated with the production of a plant that can reproduce itself from the seed (hybrid seed segregates, inbred seed does not).

There remains a need for a grain that has added nutritional value due to reduction of the phytic acid produced in the plant. There also remains a need for a method of making seed lethal to prevent volunteer plants developing. There remains a need for an inducible lethal trait in seed of a number of plant species.

Summary of the Invention

It is an object of the present invention to provide seed, specifically maize seed, with very low phytate content.

It is another object of the present invention to provide grain, preferably nonmaize grain, that have added nutritional value due to reduction of the phytic acid produced in the plant.

It is yet another object of the invention to provide a nongerminable seed which contains at least one genetic construct which is under the control of and operably linked to a non-natural inducible promoter or other genetic element wherein the at least one genetic construct, when induced, produces a germinable seed. The seed of the present invention contains a nongerminable trait or phenotype as well as a genetic element which is under the control of an inducible promoter such that when the promoter is induced, the non-germinable trait is corrected or overcome and the seed will germinate

It is another object of the invention to provide a germinable seed which contains at least one genetic construct which is under the control of and operably linked to a non-natural inducible promoter or other genetic element wherein the at least one genetic construct, when induced, produces a nongerminable seed. The seed of the present invention contains a germinable trait or phenotype as well as a genetic element which is under the control of an inducible promoter such that when the promoter is induced, the germinable trait is destroyed or overcome and the seed will not germinate.

A further object of the present invention is to provide nucleic acid molecules (sequences) which code for proteins involved in the synthesis of phytate as well as the phytate encoded for and antibodies to same.

An object of the present invention is to provide vectors (expression and cloning), and methods for using same, for making the seed of the present invention which preferably contain the phytate encoding nucleic acid sequences, or allele, or truncated, substituted, or inserted variants or fragments thereof.

A further object of the present invention includes providing methods of producing inducible nongerminable seed and inducible germinable seeds..

Yet a further object of the present invention is a method of preventing volunteer plants from developing from fallen seed which includes planting and growing the germinateable mature seeds of the present invention and applying an activator to the plants produced therefrom, to produce daughter seeds or embryos which will not germinate.

Still further objects and advantages will become apparent from a consideration of the present description and accompanying drawings.

Brief Description of the Drawings

FIG. 1 shows a binary representation of a construct according to the present invention developed for use in dicots and those monocots capable of Agrobacteruim transformation, the construct contains the right T- DNA border, the NOS promoter, the NPTII marker gene and the NOS terminator, the inducible promoter, in this instance the GSTII promoter sequence, and the phytate gene in the sense orientation and the NOS terminator and the left T-DNA border.

FIG. 2 shows a binary representation of a construct according to the present invention developed for use in dicots and those monocots capable of Agrobacteruim transformation. The construct contains the right T- DNA border the Nos promoter the NPTII marker gene and the NOS terminator, the inducible promoter in this instance promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 and the sequence shown in Figure 5 therein (this patent incorporated by reference herein), deposited with the American Type Culture Collection (ATCC),

Manassas VA , Accession No. 67804, and the phytate gene in the antisense orientation and the NOS terminator and the left T-DNA border.

FIG. 3 shows a construct with the promoter. In this instance the promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 and the sequence shown in Figure 5 therein deposited with the American type Culture (ATCC), Manassas, VA, Accession No. 67804, the phytate gene of the present invention and the NOS terminator. The phytate gene is in the sense orientation.

FIG. 4 shows a construct with the GSTII promoter sequence, the phytate gene of the present invention and the NOS terminator. The phytate gene is in the anti-sense orientation.

FIG. 5 shows the prior art sequence of the nucleotide sequence of the 5-2 gene promoter from the gene designated 52.411 in U.S. Patent No. 5,608,143.

FIG. 6 shows an example of data obtained from 3 inbred lines screened for phytate content, plotted as a frequency distribution curve.

FIG. 7 shows data taken from a low-phytate mutant segregating for low phytate (high-phosphorus) content.

FIG. 8 shows the nucleic and amino acid sequences of myo-inositol 1-phosphate synthase, Accession AF056326.

FIG. 9 shows a linear map of the published sequence of Zea Myo-inositol 1-phosphate synthase from base 86 to base 1620

Detailed Description

EMS (Ethyl Methane Sulfonate) has been used to induce mutant phytic acid genes in maize to produce low-phytate seed. For example, a low phytic acid maize mutant, lpa1, has been produced by Raboy (U.S. Patent No. 5,689,054). In some instances seed may be sufficiently low in phytate as to prevent germination of the seed. In other cases low phytate seed can be found that germinate normally.

From these mutated plants the gene of the present invention is isolated

and cloned in a repeatable manner. The cloned gene encodes for an enzyme that affects the production of phytic acid in the seed. The gene of the present invention includes allelic, truncated, deleted and substituted variants of the cloned gene as well as useful fragments, such as those which may be used to down-regulate the gene in an anti-sense manner, as described herein. The present invention provides a transformation vector containing the new gene operably linked to an inducible promoter so that the lethal effects of the gene can be managed, exploited or controlled.

This vector is useful in crops which are not hybrids such as soybeans, wheat, barley, maize, canola and sunflowers because the vector can be used to make seed with a very low phytic acid content and also the seed can only germinate in highly controlled conditions following induction. This vector of the present invention, when present in a plant, has the following several advantages: (i) it is useful to farmers because it eliminates volunteer plants in the next season by rendering the seed incapable of germinating, (ii) it is useful to a seed company because it maintains germplasm security by rendering the seed incapable of being reproduced for breeding purposes, (iii) it prevents farmer saved seed by rendering the seed incapable of being reproduced for future years, and furthermore, (iv) it is useful in the feed and milling industry because it adds nutritional value to the feed or milled products by being low in phytic acid content. This invention is particularly useful in the flour grains and the feed grains. These include, but are not limited to corn, wheat, soybeans, sunflower, oats, rye and the like.

The present invention provides, therefore, methods of seed security which includes providing a low-phytate, non-germinating daughter seeds or embryos which have been altered to include a functional phytate coding gene which is operably linked to, and under the control of, an inducible promoter.

Targeting and isolating gene

Genes of the present invention may be isolated, for example, by crossing with a mutant maize corn plant that has a genetic mutation that decreases the levels of phytic acid in the seed. From this gene, the wild-type

or native gene, and the mutant gene, can be identified. Both the native phytic acid gene or the mutated phytic acid gene can be employed in the present invention.

In one embodiment, the present invention provides methods to make and use seed, as well as seed and plants therefrom, wherein the phytic acid content of the seed is eliminated or severely reduced by insertion of an antisense construct of genes or fragments thereof which encode, in the sense direction, proteins involved in the synthesis of phytic acid which, even when operably linked to a weak promoter, are expected to at least severely reduce the phytic acid levels of the seed.

In another embodiment, the present invention provides methods to make and use seed, as well as plants therefrom, wherein the phytic acid content of a low-phytic acid mutant containing seed is restored by insertion of a sense construct of the native gene or fragments thereof, i.e., native gene which has been mutated to produce the low-phytic acid phenotype, which, even when operably linked to a weak promoter, are expected or known to increase the phytic acid levels of the seed.

One method of isolating the phytate encoding nucleic acid sequence of the present invention is through the use of transposon-tagged mutants which allow for cloning of the gene, by means known in the art. The transposable element tags the gene, in other words the sequences flanking the transposon insertion site are part of the gene of interest. According to the present invention, which is exemplified herein as maize, a low-phytic acid mutant inbred line is crossed to a line containing the mu1 mutator gene and a low phytic acid mutant gene is identified by the mu1 element insertion into the gene of interest. The mu1 used in the exemplified embodiments of the present invention is a part of the mu1-9 family sharing the ~20-bp terminal inverted repeat structure. Multiple copies of the transposon are contained in the mutator lines, which are aggressive mutagens in maize. Most mu insertion events give a null phenotype because the transposable element insertion does not result in altered essential proteins. With targeted transposon tagging, the insertion event is targeted to specific known

mutations, such as the low phytic acid gene. Transposon events occur in the range of ~10^{-4} to 10^{-6} insertions per locus per chromosome tested. To identify when the phytic acid gene has a DNA transposon inserted therein requires that there is identification of a mutant phenotype from the insertion event and restoration of partial or full function in the gametes from the excision events. The tagged mutant can then be crossed with a low phytic acid mutant plant carrying the recessive low phytic acid gene. If the transposable element has landed or inserted in the gene of interest then the grain from the cross can be identified as low phytic acid grain or low phytate grain. The gene is recessive and its effects are only present if the parents both carry the low phytic acid gene. The allelism is checked in a similar breeding crossing manner with similar known low phytic acid mutants.

To find the transposable event requires several hundred thousand progeny if the less active elements are used. Use of mu1 substantially reduces the required number of progeny that need to be screened for the grain trait. Depending on the transposable element used it maybe useful to backcross the inbred having the targeted mutant gene. Backcrossing was used in the present exemplification of the invention described herein because the mu1 line was used. The mu1 line can be identified as having lost mutator activity through the backcrossing procedure by monitoring the reporter gene of the anthocyanin pathway which produces a purple coloring in the seed. The backcrossing to the standard maize inbred simplifies the segregation analysis because the copy number will be greatly reduced. The allelism is checked with similar known mutants. The mu1 element is cloned using the transposon element as a probe to clone the mutant alleles or alternatively to probe for the native wildtype gene. This method identifies the low phytic acid native and mutant genes in seed, which may then be isolated, purified and sequenced, by means known in the art.

The method for finding mutable alleles, as an efficient means for cloning functional genes, requires a mutant tagged with an insertion element, a clone of the element, assays for verifying the clone recovered, and simple genetics tests. The loss of reporter gene activity is used to show loss of

mutator activity. In general, the process involves a process termed co-segregation. This involves selecting and harvesting tissue for the molecular cloning; preparing DNA from the progeny of crosses of diverse lines; and making samples from several m/m (homozygous recessive mutant genes) and several M/M (homozygous wild-type genes) individuals from each line. Next, southern blots are prepared from DNA taken from the mu-tagged material after digesting the samples with several 5-methyl cytosine-insensitive enzymes that do not digest the mu1 element that is tagging the mutant; bands are located and identified which are present in all m/m individuals that are not present in the M/M individuals; and a DNA pool of the m/m individuals are prepared and three samples from each diverse line are sampled. The darkest band as well as the only shared, diploid copy fragment are searched for and selected. This process of co-segregation will identify the mu-element which is tagging the mutant-gene. Results were verified by matching the fragment with the segregation analysis. The fragment is identified from the southern blots by locating the line with the fewest number of segregating bands that has no evidence of a co-migrating band. A large sample of m/m and M/M progeny of this line are analyzed to evaluate the statistical significance between the band and the phenotype. Various restriction enzymes are used for this confirmation. Significant samples in this population test are subjected to digestion by several enzymes, in order to screen for enzymes that do not digest the bands. This is done as a double digestion with the initial restriction enzyme as well as enzymes which poorly digest the bands, in combination with high resolution agarose gels. Then the m/m digest pool of DNA which was not digested is multiplied and the DNA is size selected and cloned.

Cloned Gene

Genes that are cloned by the process described above are genes which encode for amino acids that produce proteins which function to alter levels of phytic acid in the plant. These genes are designated as phytate genes (phytic acid genes).

Inducible promoter

A number of compounds have use in the control of gene expression in plants. The inducer of gene expression must be safe and not have detrimental effects on the desired agronomic traits of the plant.

Natural products, including hormones, and other chemicals, are known which affect gene expression in plants. Plant growth regulators which include AAA , ethylene, absisic acid, auxin, salicylic acid and other plant hormones all affect gene expression. These natural chemicals appear to induce gene expression. Hormones, whether natural or synthetic, which affect the gene expression in plants, can be used with the present invention. However, care must be taken to avoid undesired activation of the growth of the plant by the co-activation of the plants internal systems or activation of lethal genes present in the plant. Clearly, use of hormones as the inducing agent must be linked to a hormone that is not going to active the metabolic system that is harmful to the end product.

Environmental inducers for regulating foreign genes include light, heat, low temperature and different gas levels in the air. These regulators can be used, individually or in combination, in accordance with the present invention. These regulators are slightly less practicable as there is not a method presently known to control very many of these environmental inducers. It is not impossible to have a cool growing season or a cloudy growing season in which these inducers may not be activated strongly or may be activated in an untimely fashion. So although these types of inducers will work with the present invention they must be selected with care.

Other plant genes have been induced by oligosaccarides which are present in wounding and pathogen infection, for example, the induction by glucan of phenylalanine ammonia lyase and chalcone synthase in soya, or the induction of a wound-like inducible inhibition gene in potato. This induction requires more effort as the plant has to be wounded to induce the foreign gene.

The inducer of choice, that is safe and has little or no effect on the

plant to which it is applied is the most useful inducer in most instances. The preferred external control of the inducer is an agent that induces the expression of the desired gene in any tissue at any time in the plant's life cycle. This requires a promoter that is activated or deactivated upon the application of the activator material such as a chemical. This regulation is accomplished by controlling this response in a number of plant species with little effect on the plant growth. The ideal activator is a chemical that is applicable with standard field equipment, in combination with something else, such as a herbicide, that is normally applied to the field to avoid repeated passes over the field . Alternatively, the material could be applied aerially by crop dusting type equipment.

At least one embodiment of the present invention uses a promoter which is known to allow (drive) the production of enzymes which protect the plant when activated by an activator, generally referred to as chemical safeners, commonly used in conjunction with herbicides. It has been determined that plants are more protected from herbicide activity when they are safened. One example of a safener is one which acts to conjugate the herbicide with glutathione. This is due to an increase in glutathione-S transferase (GST) activity increases the mRNA of the GST in the safened plants. Thus this safener treatment increases the gene product but does not negatively effect the plant.

It has been shown that corn can be safened by a wide variety of agents, including, but not limited to, naphalic anhydride, N, N-dially-2,2-dichloroacetamide or cyometrinil when sulfonylurea herbicides are employed. The rates of the metabolism of chlosulforon and metsulfuron methyl increase within hours of the application of the safener. In the present invention, it is the promoter of the GST enzyme which is used as the switchable promoter linked to the phytate gene of the present invention. Switching is then achieved by use of a switch-inducing chemical agent.

The gene-inducer useful in the present description can be any number of inducing or activating agents. Clearly the inducing agents in either US Patent No. 5,608,143 (incorporated by reference) and/or WO 90/08826,

(incorporated herein by reference) entitled, "Gene Switch" are useful. The method of using an inducing agent to switch on a gene is known in the art and can be implemented according to the teaching of the present invention. These methods are also described in WO93/09237.

The preferred promoters of the present invention are those which respond to safeners used in herbicide formulations. In nature these promoters and their associated genes are induced by the safener in the herbicide to protect the plant from the damage that the herbicide can inflict on the plant in the absence of the safener. In the present invention, these promoters and heterologous genes are preferably activated and switched on or off by the safener. The safener is preferably applied in a herbicide and a marker gene can make the plant resistant to such herbicide if it is not already resistant. Alternatively the chemical, such as the safener, can be applied separately to the plant. This may be useful if the herbicides primary application time is significantly earlier then seed development.

The present invention provides therefore, methods to make and use seed, as well as seed and plants therefrom, wherein the phytic acid content of the seed is eliminated or severely reduced by insertion of an antisense construct of genes or fragments thereof which, in the sense direction, encode proteins or fragments of proteins involved in the synthesis of phytic acid which, even when operably linked to a weak promoter, are expected to at least severely reduce the phytic acid levels of the seed; the construct being operably linked to a promoter or other genetic element which is induced by a chemical or environmental factor, preferably a safener, such that application of the chemical or environmental factor to the plants produced from the seed produce seeds with a severely reduced amount of phytic acid when compared to the original seed.

Preferably, the seed produced from the plant which has received the factor contains less than about 5% of control phytate content by weight , more preferably less than about 2% of control phytate content by weight, more preferably less than about 1% of control phytate content by weight phytic acid than the seed which produced the plant which has received the factor.

One of ordinary skill will appreciate that common means of measuring phytic acid requires destruction of the seed such that these comparison values are most commonly average values of a sampling of seed produced as described herein. Moreover, one aspect of the present invention is to provide non-germinable seed after induction of the parent plant, irrespective of the decrease in phytic acid amount as compared to the parent seed.

In another embodiment, the present invention provides methods to make and use seed, as well as plants therefrom, wherein the phytic acid content of a low-phytic acid mutant containing seed is restored by insertion of a sense construct of the native gene or fragments thereof, i.e., native gene which has been mutated to produce the low-phytic acid phenotype of the low-phytic acid mutant, which, even when operably linked to a weak promoter, are expected or known to increase the phytic acid levels of the seed; the construct being operably linked to a promoter or other genetic element which is induced by a chemical or environmental factor, preferably a safener, such that application of the chemical or environmental factor to the plants produced from the seed produce daughter seeds with an increased amount of phytic acid when compared to the original seed. Preferably, the seed produced from the plant which has received the factor contains more than about 5% of control phytate content by weight, more preferably more than about 8% by weight, more preferably more than about 10% by weight phytic acid than the seed which produced the plant which has received the factor.

One of ordinary skill in the art will appreciate that the low or reduced phytic acid plants and seed of the present invention may be produced by transformation with nucleic acid molecules encoding phytase, preferably operably linked to an inducible promoter of the present invention, either in conjunction with the phytate genes and variants described herein, or alone. Expression of any of these phytases (enzymes of phytic acid degradation) during grain development may be used to degrade phytic acid being synthesized in the developing seed. Phytase encoding sequence are known, such as have been published by the NCBI, and elsewhere, under any of the following accession numbers: P34752 ,

gi|464382|sp|P34752|PHYA_ASPNG [464382]; P34753 , gi|464381|sp|P34753|PHYA_ASPAW [464381]; 2599490 (AF029053) phyb13 precursor [Bacillus subtilis] gi|2599490 [2599490] ; 1IHP gi|2981783|pdb|1IHP| [2981783]; 3150040 (U85968) gi|3150040 [3150040]; 2943981 (U92439) phytase [Enterobacter cloacae] gi|2943981 [2943981]; P34754 gi|464385|sp|P34754|PHYB_ASPNG [464385]; P34755 gi|464384|sp|P34755|PHYB_ASPAW [464384]; P07102 gi|130735|sp|P07102|PPA_ECOLI [130735]; 2108356 (U59802) phytase [Talaromyces thermophilus] gi|2108356 [2108356]; 2108354 (U59804) phytase [Aspergillus fumigatus] gi|2108354 [2108354]; 2108352 (U59803) phytase [Emericella nidulans] gi|2108352 [2108352]; 2300890 (A46793) gi|2300890|gnl|PID|e306401 [2300890]; 2300889 (A46793) gi|2300889|gnl|PID|e306184 [2300889]; 2300887 (A46791) gi|2300887|gnl|PID|e306400 [2300887]; 2300885 (A46789) gi|2300885|gnl|PID|e306183 [2300885];2300883 (A46787) gi|2300883|gnl|PID|e306182 [2300883]; 2300881 (A46785) gi|2300881|gnl|PID|e306399 [2300881];2300879 (A46783) gi|2300879|gnl|PID|e306181 [2300879]; 2148991 (U60412) phytase [Aspergillus terreus] gi|2148991 [2148991]; JN0715 3-phytase (EC 3.1.3.8) B precursor - Aspergillus ficuum gi|542378|pir||JN0715 [542378]; JN0890 acid phosphatase (EC 3.1.3.2) precursor - Aspergillus awamori gi|542377|pir||JN0890 [542377]; JN0889 3-phytase (EC 3.1.3.8) A precursor - Aspergillus awamori gi|542376|pir||JN0889 [542376]; JN0656 3-phytase (EC 3.1.3.8) A precursor - Aspergillus niger gi|484414|pir||JN0656 [484414]; JN0482 3-phytase (EC 3.1.3.8) A - Aspergillus ficuum gi|419906|pir||JN0482 [419906]; PQ0641 3-phytase (EC 3.1.3.8) - Aspergillus ficuum (fragments) gi|542374|pir||PQ0641 [542374]; S33278 phytase P2 - Escherichia coli (fragment) gi|421153|pir||S33278 [421153]; B36733 acid phosphatase (EC 3.1.3.2) precursor - Escherichia coli gi|96267|pir||B36733 [96267]; S18408 alkaline phosphatase (EC 3.1.3.1) - rat gi|111353|pir||S18408 111353]; 1943870 (U59806) phytase [Thielavia heterothallica] gi|1943870 [1943870];1943868 (U59805) phytase

[Aspergillus terreus] gi|1943868 [1943868]; 1938256 (U75531) phytase [Zea mays] gi|1938256 [1938256]; all sequences and text of US patent 5593963 , including, gi|1831488|pat|US|5593963|20 1831488]; 408990 phytase {EC 3.1.3.26} [Aspergillus ficuum, Peptide, 441 aa] [Aspergillus ficuum] gi|408990|bbs|130910 [408990]; 235916 gi|235916|bbs|58159 [235916]; 235915 gi|235915|bbs|58160 [235915]; 2393 gi|2393 [2393]; all sequences and text of US-5436156 , including gi|912286|pat|US|5436156|32 [912286]; 583196 (A19452) gi|583196 [583196];583194 (A19451) gi|583194 [583194];166521 (M94550) gi|166521 [166521]; 166519 (L02421) phytase [Aspergillus niger] gi|166519 [166519]; 166482 (L02420) acid phosphatase [Aspergillus niger] gi|166482 [166482]; 304097 (L20567) phyB Aspergillus niger] gi|304097 [304097]. Methods and constructs useful in the present invention may also be found in U.S. Patent No.s 5,770,413 and 5,593,963.

In a further embodiment, the construct and promoter or other genetic element are operably linked to a marker gene as are known in the art.

In one embodiment of the present invention, the plants and seed described in the embodiments of the invention are any one of maize, soybeans, rice, oats, sunflower, wheat, barley, forage grasses, and the like

Constructs

The present invention includes a construct that has a promoter associated with a phytic acid gene, the promoter is under external control such that when activated makes the seed so low in phytic acid that the seed is unable to germinate (ie it can not form a plant that matures). This construct can be transformed into any number of plants that are transformable. The positioning of the gene within the construct determines whether the gene is going to be up regulated, the sense orientation, or down regulated, the antisense orientation.

Figure 1 shows a binary representation of a construct according to the present invention. This construct is developed primarily for use with Agrobacterium transformation. Thus it is principally developed for use in dicots and those monocots capable of Agrobacteruim transformation. The

construct contains the right T- DNA border the Nos promoter the NPTII marker gene and the NOS terminator, the inducible promoter in this instance the GSTII promoter sequence and the phytate gene in the sense orientation and the NOS terminator and the left T-DNA border. This construct is ideal for soybeans and like cultivars. The marker gene can be selected to be a herbicide resistant gene such as glyphosate resistant gene.

Figure 2 shows a binary representation of a construct according to the present invention developed for use in dicots and those monocots capable of Agrobacteruim transformation. The construct contains the right T- DNA border the Nos promoter the NPTII marker gene and the NOS terminator, the inducible promoter in this instance the promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 the sequence shown in Figure 5 and the phytate gene in the antisense orientation and the NOS terminator and the left T-DNA border. This safener is applied to activate the promoter and thus express the product encoded by the gene by application of the herbicide containing the safener or just by safener application.

Figure 3 shows a construct with the promoter in this instance the promoter region from the gene encoding the cDNA clone 5-2 described in U.S. Patent No. 5,608,143 the sequence shown in Figure 5 therein this patent incorporated by reference herein, deposited with the American Type Culture Collection (ATCC), Manassas, VA, Accession No. 67804. The phytate gene of the present invention and the NOS terminator. The phytate gene is in the sense orientation. Thus when the activator is applied the promoter switches on and the gene will express the amino acids it encodes for.

Figure 4 shows a construct with the promoter in this instance the GSTII promoter sequence and the phytate gene of the present invention and the NOS terminator. The phytate gene is in the anti-sense orientation.

These constructs in their various forms can be transformed into maize, soybeans, rice, oats, sunflower, wheat, barley, forage grasses, and the like. The present invention includes constructs which have been optimized for expression in each of these plants, such as, for example, with respect to

codon usage, promoters, etc. and any other expression-enhancing process as well as optimised for producing an active enzyme or optimal antisense or cosuppression effects. Additionally, the constructs of the present invention can be transformed into plants that are already transformed or already mutants. For example this gene construct can be used to make the existing low phytic acid mutants externally regulated by application of the activator. The promoter in the construct can act to induce the gene to express or the activator can stop the expression of the gene by inducing the promoter to shut down. Likewise, the orientation of the gene in the construct may be in the sense or antisense direction, with sense orientation up regulating the gene expression and antisense orientation down regulating the gene expression. Thus the same effect can be produced by switching the promoters from inducible on to inducible off, or changing the orientation of the gene.

Transformation

Representative diagrams of vectors in accordance with the present invention are shown in the first four figures. The first two figures show a construct that is adapted for Agrobacteruim transformation. The second two figures show a plasmid for use with other transformation techniques such as micro particle bombardment. These transformation techniques are well known to those of skill in the art. Additionally, these constructs are excellent for use with the whiskers transformation system taught in U.S. Patent No. 5,302,523, and U.S. Patent No. 5,464,765 incorporated herein by reference.

EXAMPLE 1

There are four preferred ways to produce the low phytic acid seed material of the present invention. The preferred method provides the farmer a seed product that is both low in phytic acid and is non-germinable so that the seed is not capable of producing a mature plant. The preferred seed of the

present invention ends farmer-saved-seed in the case of cultivars which are not hybrids. Presently, most canola and wheat and soybeans are not sold as hybrids though both wheat and canola have ongoing research to develop hybrid capabilities. If the plant is not normally commercialized as hybrid seed but is instead seed like wheat and soybeans then the material according to the present invention is very readily produced.

Transformant Breeding with inbred material

If the promoter is induced to turn the gene on, and the gene is in the antisense orientation then application of the activator will produce seed with extremely low levels of phytic acid. Such seed would not germinate. The activator is applied preferably when the grain is filling or slightly prior thereto. The activator is preferably in the form of a safener that can be in a herbicide if the promoter is the GSTII or other safener-induced promoter. Thus, during seed increase the seed is not sprayed with the activator. This allows the production of seeds with normal phytic acid levels. However, when the seed is sold to the farmer for low phytic grain production then the activator must be applied to the plant to produce a seed with low or no phytic acid. Because of the low phytate in the seed, the activator will also make the seed unable to germinate. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and seed can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate; the mature seed containing a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which, in the sense direction encode phytic acid or fragments thereof or allelic variations thereof, the nucleic acid molecules being positioned in the construct in the antisense direction to the promoter, such that when the promoter is activated by an activator in a plant grown from the mature seed, a product developing seed or embryo is produced which will not germinate. The present invention provides the plant produced from the mature seed and the methods of reducing the

growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

If the promoter is induced to turn the gene off, and the gene is in the sense orientation, then the plant, absent the construct containing the promoter and gene, would have to contain a mutant form of the gene which produces very low levels of phytic acid in the seed such that the seed, absent the construct containing the promoter and gene, does not germinate. Application of the activator to the plant produced from the mature seed containing the construct of this embodiment will produce non-germinable developing seed or embryo which contain extremely low levels of phytic acid. The activator is preferably in the form of a safener in a herbicide if the promoter is or contains a fragment of the promoter shown in Figure 5. The activator is preferably a substituted benzenesulfonamides. Thus during seed increase, i.e., growth of the progeny of the mature seed of this embodiment of the invention for increasing the number of mature seed of this embodiment, the plant produced from the mature seed of this embodiment is not sprayed with the activator. However, when the mature seed of this embodiment is being grown to provide seed or grain for farmers or end-users, for production of low phytic products, then the activator must be applied to the plants produced from the mature seed of this embodiment of the invention to produce the low-phytic, non-germinable developing seed (embryo or child seed). This will result in the production a seed with very low phytic acid which will also make the seed non-germinable. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate wherein at least a portion of the sufficient amount of phytic acid is produced from a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which encode phytic acid, such that when the promoter is activated by an activator in a plant grown from the mature seed, a developing seed or embryo is produced by the plant which will not germinate. The mature seed

of this embodiment, absent the construct, does not produce suffiient phytic acid to germinate. The present invention provides the plant produced from the mature seed and the methods of reducing the growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

If the promoter is induced to turn the gene on, and the gene is in the sense orientation, then the plant would have to contain a mutant gene that is producing extremely low levels of phytic acid in the seed. Such seed would be unable to germinate. Application of the activator in this embodiment will restore germinability as increased levels of phytic acid will be produced. The activator is preferably in the form of a safener in a herbicide if the promoter is or contains a fragment of the promoter shown in Figure 5. The activator is preferably a substituted benzenesulfonamides. During seed increase, i.e., growth of the progeny of the mature seed of this embodiment of the invention for increasing the number of mature seed of this embodiment, the plant produced by the mature seed of this embodiment is sprayed with the activator. However, when the seed of this embodiment is being grown to produce seed or grain to the farmer or other user as low phytic containing seed or grain then the activator must not be applied. This will result in the production a seed with very low phytic acid which will also make the seed non-germinable. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate wherein at least a portion of the sufficient amount of phytic acid is produced from a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which encode phytic acid, such that when the promoter is activated by an activator in a plant grown from the mature seed, a developing seed or embryo is produced by the plant which will germinate. The mature seed of this embodiment, absent the construct, does not produce suffiient phytic acid to germinate. The present invention provides the plant produced from the

mature seed and the methods of reducing the growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

If the promoter is induced to turn the gene off, and the gene is in the antisense orientation, then the plant, absent the promoter and operably linked gene, would have to contain a normal or native gene that is capable of producing normal levels of phytic acid in the seed. Such seed, with the promoter and operably linked gene, will not germinate. Application of the activator will restore the production of phytic acid and hence the seed with the promoter and operably linked gene of this embodiment will germinate normally. The activator is in the form of a safener in a herbicide if the promoter is or contains fragments shown in Figure 5. The activator is preferably a substituted benzenesulfonamides. Thus during seed increase, as described above, the plant produced from the mature seed of this embodiment is sprayed with the activator. However, when the seed is sold to the farmer or other end user, as described above, as low phytic then the activator must not be applied. This will result in the production of a seed with low phytic acid and thus low phytate in the seed and the absence of the activator will also make the seed non-germinable. Thus the soybean or other plant species' seed will not grow the following season as volunteer plants and can not be saved for use in the following year for planting purposes.

In this embodiment, the present invention provides a mature seed which contains a sufficient amount of phytic acid to germinate; the mature seed containing a genetic construct which contains an inducible promoter operably linked to nucleic acid molecules which, in the sense direction encode phytic acid or fragments thereof or allelic variations thereof, the nucleic acid molecules being positioned in the construct in the antisense direction to the promoter, such that when the promoter is activated by an activator in a plant grown from the mature seed, a developing seed or embryo is produced which will germinate. The mature seed of the present embodiment, absent the construct, is capable of making at least enough phytic acid to germinate. The antisense arrangement o the nucleic acid

molecles in the construct reduce the amount of phytic acid to the extent that the developing seed or embryos from a plant produced from the mature seed will not germinate unless activator is applied to the plant. The present invention provides the plant produced from the mature seed and the methods of reducing the growth of volunteer plants and of reducing the amount of germinable saved seed which include applying an activator to the plant.

Other combinations of gene orientation and promoter inducibility and other activators are known. The safener or a pesticide activator is preferred as it decreases the time and expense of covering the field more then once. In other words it provides the dual utility of acting as a herbicide or pesticide and inducer to the gene(s).

Transformant Breeding with hybrid material

The number of gene orientation and mutant and wildtype combinations useful in the present invention increases when the plants are hybrid plants. A method of hybrid transformant breeding employs hybrids formed with two mutant low phytic acid inbreds, such as are described above, at least one of the mutants carrying a genetic construct which contains a transforming gene, or allelic, substituted, deleted or truncated variation thereof which was capable of encoding amino acid sequences necessary to produce phytic acid, preferably the construct contains the gene in the sense direction encoding for the wildtype phytic acid. During production, the lethal mutant carrying the transformed wildtype gene is exposed to the gene inducing spray during the period of seed production. This induces the promoter and the sense gene is turned on and the gene produces the necessary phytic acid to maintain the germination ability of the seed. This acts as the protector of the line and also as the maintainer of the line. Preferably, the producer of the seed applies the activator as opposed to the end-user, or farmer. The end-user or farmer need only plant the mature seed. When the inducible promoter is activated by a safener, as in the preferred embodiments, one of skill will appreciate that a number of plant herbicide may carry the activator that will induce the gene and care must be taken to limit or discourage the use of herbicides that

include the safener during the end-user or farmers production of the hybrid seed. Alternatively, herbicides could be formulated which do not contain the activator of the present invention to be applied separately from the activator/saffener. In this case, it is preferable that the herbicide and activator be applied separately.

Another method of the present invention uses a wildtype plant transformed with a wildtype gene, that, when positioned in a sense direction, encodes for normal levels of phytic acid. In this embodiment of the present invention, however, the wildtype gene is in the antisense direction and operably linked to an inducible promoter, as described herein. The gene is positioned in the antisense position to knock out expression of the wildtype gene in the plant when the plant is exposed to the activator chemical. This antisense gene is induced in the field by application of inducing chemical or environmental factor prior to production of grain or during development of the developing seed or embryo.

Alternatively, a natural mutant plant having lethal phytic acid levels may be used and is modified to include the wildtype gene in the sense orientation operably linked to an inducible promoter, as described herein, inserted such that when the promoter is induced in the plant, it over-expresses this gene and is able to produce daughter seed or embryos which will germinate. The inducible promoter is shutdown by the chemical. The chemical activator is applied, such as by spraying, on the plant by the end-user or farmer prior to the onset of senescence of the plant.
In this way, the mature seed contains the phytic acid genes needed to produce phytate levels needed during germination, but the gene is turned off and the chemical is not applied and the wildtype gene is not expressed.

The present invention provides therefore, a gene switch operably linked to nucleic acid molecules encoding amino acid sequences necessary to produce phytic acid. The direction of the gene within the construct and the specific promoter switch may be selected according to the present invention by one of ordinary skill depending on the genetic and phenotypic background of the tissue to be transformed as well as the desired product (daughter seed

or embryo) to be produced.

EXAMPLE 2

The method of producing low-phytate mutants useful for transposon tagging is a known method called mutagenesis. The process is outlined in the Neuffer paper Maize Genetic Newsletter 45:146. Mutations were induced in the inbred line by treating pollen with ethyl methane sulfonate in paraffin oil according to the procedure described by Neuffer (1974). This treatment was performed on a number of inbreds from the various plant genotypes of cereal. This example will focus on the development of maize low-phytate mutants by this process. This mutagenesis process has been used to make a number of cereal mutants.

The general steps of the process of the present invention include treating inbred pollen (in this case maize) with ethyl methane sulfonate hereinafter "EMS". Inbred pollen is placed in EMS in oil for 45 minutes. A paint brush is used and the pollen is brushed on to the silks of a receptive corn ear. This forms the Mutant-1(M1) seed. Such seed are grown and self-pollinated to produce the Mutant-2 (M2) kernels. The resulting M2 kernels are tested for the low phytate phenotype.

A preferred maize mutant of the present invention which may be used as the basis for transposon tagging and phytic acid gene isolation is the Maize (Zea Mays) inbred line EX1965PY deposited in the ATCC (American Type Culture Collection, Manassas, VA) under conditions of the Budapest Treaty, on July 7, 1998, Designation No. 20 3034. Alternatively, *Zea maize* seeds of lpa1 (lpa1-1) and lpa2 (lpa2-1) mutant homozygotes described in U.S. Patent No. 5,689,054, which were deposited on August 15, 1996, under the terms of the Budapest Treaty at the American Type Culture Collection (ATCC) and have been assigned Accession No.s ATCC 97678 and ATCC 97679, respectively, may also be used. Preferred phytic acid genes of the present invention are those obtainable from these deposited materials, preferably by transposon tagging methods known in the art. Further preferred

nucleic acid sequences of the present invention include SEQ ID NO:1, a sequence encoding the protein shown in SEQ ID NO:2, and Figures 8 and 9, and sequences which have been published by NCBI (National Center for Biological Information) under Accession No.s g3108052 and g3108053. Nucleic acid sequences which encode the amino acid sequences of SEQ ID NO:2 and those shown in Figures 8 and 9, are also included as are allelic, substituted, deleted or truncated variations thereof which was capable of encoding amino acid sequences necessary to produce phytic acid.

EXAMPLE 3

The HVPE method is a common test for low phytate mutants. (Raboy, Maydica 35:383 (1990)). The method relies on differential migration of phosphorus compounds. After electrophoretically fractionating the compounds, a chromatogram allows a semi-quantitative assessment of the phytic acid relative to other compounds. An alternative method involves screening for higher levels of inorganic P in the grain. For example grain samples can be ground (to pass a 2 mm screen in A Wiley mill) followed by addition of either 50 mg of grain germ or 1 gram of endosperm in 15ml of 0.4 M HCL in 0.7 $Na_2 SO_4$. Phytic acid precipitates as an iron salt. Phosphorus in the ferric phytate precipitates and total P are determined. Phytic acid P (mg) are converted to phytic acid by a conversion factor 3.5.

The principles of phytate measurement are known. In the method employed in the present study, a solution of 5-sulfosalicylic acid and $FeCl_3$ (Wade reagent) forms a pink chromophore. Phytic acid binds iron in this solution decreasing the level of the pink color. The measurement of this loss of color can be used as an indication of phytic acid levels. Since the blank contains no phytate, all readings of samples that do contain phytate will be negative numbers. If there is too much phytate, however, the iron-phytate complex can precipitate as a milky white substance. In this case the pink color will not be present but the milky white matter will absorb light and result in falsely high readings. Thus some visual observation may be necessary.

This may necessitate using a smaller aliquot (less than 25 microliters) of the corn extract if the corn variety has high levels of phytate.

A rapid screening procedure, such as described as follows, may be used to score for putative low phytate seeds. In this procedure, a single edge razor is used to cut the kernel tip cap off just behind the black layer. The cut should transect the scutellum at a point at or near the radicle tip. Usually, 8 representative kernels were selected from each ear. The kernels are then placed, cut surface up, on a microplate that has the surface covered with cellophane tape (sticky side up). The staining procedure was completed after dissection of at least 100 families.

Staining was done with the use of a repeating pipette to place a 10 microliter drop of Wade reagent (as described below) on the cut surface of each kernel. After a few minutes the color disappeared as the phytic acid from the scutellum binds the iron in the Wade reagent. Observations were made for families that segregate for slower disappearance of the pink color relative to the others (perhaps 5 % of the total). These slower families were re-analyzed by the quantitative procedure described herein.

Phytate was quantified as follows. Individual kernels (7 to 10 from each family) were crushed in steel plate of a Carver hand-pump press (best results obtained when wells of plate lined with glycine paper and crushed with about 5000 lbs. pressure) and placed into 1.5 ml microcentrifuge tubes. 1 ml of 0.65 N hydrochloric acid was added and allowed to stand overnight. The combination was mixed by inversion the next day and allowed to settle for 5 minutes. A 15 microliter aliquot of the supernate was added to a microfuge tube with 100 microliters of buffer A (as described herein) and mixed. Low phytate mutants turn a very blue color due to the high phosphorus levels of the seeds. Mutants were generally retested the following day. The 0.65 N HCl extraction solution was made by adding 216 ml of 12.1N HCl to 3784 ml water. Reagent A was made fresh daily and included 2 parts (by volume) deionized water, 1 part (by volume) ascorbic acid solution, 1 part (by volume) ammonium molybdate solution and 1 part (by volume) H_2SO_4 solution. Ammonium molybdate solution was made by adding 25 g $(NH_4)_6 MO_7O_{24}$ x

$4H_20$ to make 1L with water. H_2SO_4 solution was made by adding 167 ml of 36 N sulfuric acid to 833 ml water. Ascorbic acid solution was made by adding 100 g L-ascorbic acid to make 1L with water. Ascorbic acid solution was stable with refrigeration for about seven weeks but only about two hours unrefrigerated.

Phytic Acid standards were prepared by means known in the art.

Maize kernel (M2) free phosphate was visually screened as follows. Kernels were selected, the phenotypes noted and placed in a multiple well crushing plate. Kernels were crushed in the multiple well crushing plate using a hydraulic press. Crushed kernels were transferred into 1.5 ml eppendorf microcentrifuge tubes. 0.5 ml of Reagent A was added. After allowing 2 hours reaction time. 0.5 ml reagent B was added. The tubes were capped and mixed by inverting. The reactions were scored visually for blueness after 1 hour, using a light box where necessary, and the bluest samples were selected as having highest phosphate. Often, the bluest samples from each family (ear) were selected and compared for final selection. Reagent A of this assay was prepared from 50 ml DMSO and 50 ml Reagent B. Reagent B was made fresh daily and prepared from 60 ml distilled water; 30 ml of 10% ascorbic solution (10 g ascorbic acid water to 100 ml total volume; the ascorbic solution was refrigerated and stable for 1 week); 30 ml of 3.5% ammonium molybdate solution (2.5 g $(NH4)_5$ Mo_7 0_{24}*$4H_20$ add water to 100 ml total volume); 30 ml of 6N sulfuric acid solution (170 ml water plus 25 ml concentrated H_2SO_4, adjust to 200 ml total volume with water).

Phytate (Red Test) was quantitatively measured as follows. Approximately 12 mature seeds were crushed in a steel crush plate of a Carver hand-pump press. Best results were obtained when wells of the plate were lined with weighing paper. Kernels were crushed with 5000 to 10000 lbs. pressure and transferred to Eppendorf tubes. 1 ml of 0.65 N HCl was added to same, allowed to sit overnight and mixed the following day by tube inversion. To assay, 200 ml of Wade-A reagent (described below) was combined with 10 ml of the above obtained corn/HCl juice extract in individual wells of a microtiter plate. Any change in color was noted and samples which

remained red were noted as low in phytate since phytate binds with iron and turns the solution white. Quantitation may be completed with a spectrophotometer measuring at 490 nm. Wade-A reagent used herein was prepared by adding 25.4 g of 5-sulfosalicylic acid and 350 mg of $FeCl_3.6H_2O$ (ground with mortar and pestel if necessary) to 1.5 L deionized water. NaOH was used to adjust the pH to 3.05 and volume adjusted with $d.H_20$ to 2L. This reagent was stable in a refrigerator for about 1 mo. 0.65N HCl was prepared by adding 216 ml HCl (12 N) to 3784 ml of $d.H_2O$.

These assays allow the selection of the desired maize plants containing the desired alleles. Further details of these procedures and embodiments according to the present invention are found in the copending PCT Application No. (Unassigned), filed July 7, 1998 entitled "Animal Feed with Low Phytic Acid, Oil Burdened and Protein Laden Grain", which is based on U.S. Provisional Application No.s 60/051,854 and 60/051,855, filed July 7, 1997, the entire contents of which are incorporated herein by reference. Other methods of testing for phosphorus are known and can be used to select plants.

Seed containing the desired levels of phytic acid are then increased. This process was employed in the present invention and a number of inbred lines were selected that carried the low phytic acid mutation. These included several low phytate inbred lines with good combining ability which were crossed together to form a hybrid that produced the grain of the present invention. Thus, the developed inbreds of the present invention were produced from stiff stalk, Lancaster and another versatile heterotic patterns so that the inbreds when crossed together with the appropriate heterotic pattern formed excellent hybrid material. It was also discovered that a number of the developed mutations of the present invention though low in phytic acid were not the same mutant as the lpa1-R and lpa2-R mutations described in U.S. Patent No. 5,689,054 as lpa1-1 and lpa2-1, respectively. Additionally, the seed were screened for germinability in standard seed germination tests. It was found that some low phytate mutants were unable to germinate whereas others would germinate normally. Only the seed with

good germination characteristics were maintained.

Figure 6 shows an example of some data obtained from 3 such inbred lines screened for phytate content. Plotted as a frequency distribution curve it is clearly evident that there were samples with very low phytate content (less than 0.5 units (weight percent)) whereas the bulk of the samples were higher in phytate content. The line known as UO95 line was selected as a starting material for its higher than average protein and oil levels. This line produces seed which are oil burdened and protein laden and which germinate normally. UO95py retains these characteristics with the low phytate levels described above. When crossed with certain other inbred lines, the resulting hybrid produces grain which are oil burdened and protein laden and contain low phytic levels.

The following table represents phytic acid contents (mg/g of seed) for mutant corn according to the present invention compared with wild-type seed.

No. Seeds	Wild-type Line	Phytic acid	Mutant Line	Phytic acid	% reduction
24	UU01	1.16	UU01-py	0.17	85.3
12	UO95	1.85	UO95-py	0.14	92.4
12	WD22	1.45	WD22-py	0.05	96.6

The following provides an example of an inbred line according to the present invention.

Inbred	Protein	Oil	Phytate	
Wild-type				
UO95	13.4	606	1.85	
UU01	12.7	2.9	1.16	
B73	11.3	4.4	---	
WD22	---	---	1.45	
Mutants	Protein	Oil	Phytate	% phytate reduction

UO95py	14.4	5.3	0.14	85.3
UU01py	12.2	3.1	0.17	92.4
B73Ipa1-R	13.2	3.2	—	—
WD22py	—	—	0.05	96.6

Protein and oil contents were measured by NIR analysis on a Dickey-John Reflectance Near Infra Red Spectrometer.

The grain of the present invention can also be used as a substitute source for the corn grain or flour used to make corn tortilla, corn meal, and cornflakes by substituting the grain of the present invention in the recipe and baking or processing as one would normally.

The grain of the present invention can also be used as a substitute for the corn wet milling industry by substituting the grain of the present invention in order to increase milling efficiency and recoverable starch content. Animal feed made as a by-product of the milling process is also substantially reduced in phytate content.

EXAMPLE 4

Methods of selecting for low phytate have been described above. Figure 7 shows data taken from a low-phytate mutant segregating for low phytate (high-phosphorus) content. Clear evidence for Mendialian segregation is apparent.

EXAMPLE 5

By crossing low-phytate mutants with a population containing mutator (transposon-tagged) it is possible to identify a rare case of a mutator-tagged gene. First, homozygous low-phytate plants are crossed with a transposon-tagged population. Next biochemical assays are used to screen the resulting seed for low-phytate content (phosphate content in this case). Using this method it is possible to identify a transposon-tagged phytate-gene specifying

the gene for the low-phytate mutation. The results of the biochemical screens are displayed in Figure 7. Here are displayed the data for the frequency distribution of seed of transposon-tagged material assayed for phosphorus content. One rare event was found in this example.

Germination tests revealed that some low-phytate mutants had acceptable germination whereas others would not germinate. Similar results may be obtained by ordinarily skilled artisans using the methods described herein.

Event	Phytate Mutants	S98 germination
mUU01py292	Low Phytate	94%
mUO95py1656	Low Phytate	51%
mUO95py1212	Low Phytate	50%
mUO95py1672	Low Phytate	33%
mUO95py2148	Low Phytate	77%
mUO95py2236	Low Phytate	48%
mWD22py1104	Low Phytate	48%
mWD22py1857	Low Phytate	29%
mWD22py2016	Low Phytate	0%
mWD22py1511	Low Phytate	78%
mTR306py510	Low Phytate	61%
TR335-E338phy	Low Phytate	0%
TR335-E75phy	Low Phytate	15%
UE95-E3645phy	Low Phytate	90%
UE95-E3654phy	Low Phytate	53%
UE95-E3746phy	Low Phytate	0%

From these preferred low phytate, non-germinating materials, it is possible, using known methods, to clone, sequence and manipulate the phytic acid mutants useful in the present invention.

* * * * * *

The entire contents of references referred to herein are incorporated in their entirety by reference.

We claim:

1. A transgenic plant containing a genetic construct comprising a heterologous nucleic acid sequence encoding for a selected gene product that regulates said plant's production of phytic acid.

2. A transgenic plant according to claim 1 wherein said nucleic acid sequence is operably linked to a promoter that is inducible by an activator.

3. A transgenic plant according to claim 1 wherein said plant also contains a mutant allele that results in the plant forming low phytic acid levels.

4. A transgenic plant according to claim 1 wherein said plant also contains a wild-type phytate gene that results in the plant forming phytic acid levels sufficient for the plant, absent said construct, to produce daughter seed which will germinate.

5. A transgenic plant according to claim 3 wherein said promoter is active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the sense direction.

6. A transgenic plant according to claim 3 wherein said promoter is not active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the sense direction.

7. A transgenic plant according to claim 4 wherein said promoter is not active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the antisense direction.

8. A transgenic plant according to claim 4 wherein said promoter is active until an activator is applied to said plant and said nucleic acid sequence is operably linked in the antisense direction.

9. A transgenic plant according to claim 3 wherein the nucleic acid sequence encodes a mutant phytate gene product obtainable from a maize mutant plant selected from the group consisting of UO95py, lpa1-1 (ATCC Accession No. 97678), lpa2-1 (ATCC Accession No. 97679) and allelic, truncated, substitution and deletion variants thereof.

10. A transgenic plant according to claim 9 wherein said gene product is obtainable by mu transposon tagging.

11. A transgenic plant according to 4 wherein said wild-type phytate gene is obtainable by mu transposon tagging phytic acid containing plants.

12. A transgenic plant according to claim 4 wherein said wild-type phytate gene is a nucleic acid sequence encoding SEQ ID NO:2.

13. A transgenic plant according to claim 4 wherein said wild-type phytate gene is SEQ ID NO:1.

14. A transgenic plant according to claim 1 wherein said plant is selected from the group consisting of maize, soybeans, rice, oats, sunflower, wheat, barley, rye, and forage grasses.

15. A transgenic plant according to claim 2 wherein said activator is a compound selected from the group consisting of: 2-chloro-N-(methylaminocarbonyl)benezenesulfonamide, 1-(n-butyl)-3-methylsulfonylurea, methyl 2-[(aminocarbonyl) aminosulfonyl]benzoate, N-isopropylcarbamoylbenezenesulfonamide, N-(aminocarbonyl)-2-chlorobenezenesulfamide and N'-[2-(n-butylaminocarbonyl)]-6-chloro-N,N-dimethyl-1,2-benezenedisulfonamide.

16. A recombinant genetic construct capable of transforming a plant, comprising

a nucleic acid sequence encoding for a gene product that regulates the production of phytic acid operably linked 3' to a nucleic acid promoter sequence.

17. A recombinant genetic construct according to claim 16 wherein said nucleic acid promoter sequence is as shown in figure 5.

18. A recombinant genetic construct according to claim 16 wherein said nucleic acid promoter sequence is inducible by application of N,N-diallyl-2,2-dichloroacetamide.

19. A recombinant genetic construct according to claim 16 wherein said nucleic acid promoter sequence is inducible by a compound selected from the group consisting of: N,N-diallyl-2,2-dichloroacetamide, benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate, naphtalene-1,8 dicarboxylic anhydride, 2-dichloromethyl-2-methyl-1,3-dioxolane.

20. A method of generating seeds comprising the following steps:

planting a mature seed containing an externally inducible recombinant DNA vector which can be activated by an activator, said recombinant DNA vector containing at least one nucleic acid sequence coding for a selected gene product that results in lethally low production of phytic acid;

applying the activator to the plant generated by said mature seed whereby the nucleic acid sequence is induced to produce said selected gene product;

harvesting the daughter seed produced from the plant after the activator has been applied.

21. A method of generating seeds comprising the following steps:

planting a mature seed containing an externally inducible recombinant DNA vector which can be deactivated by an activator, said recombinant DNA vector containing at least one nucleic acid sequence coding for a selected gene product that results in lethally low production of phytic acid;

applying to the plant generated by said mature seed, the activator whereby

the nucleic acid sequence is induced to stop encode said selected gene product;

harvesting the daughter seed produced from the plant after the activator has been applied.

22. Daughter seed produced by the transgenic plant according to claim 5.

23. Daughter seed produced by the transgenic plant according to claim 6.

24. Daughter seed produced by the transgenic plant according to claim 7.

25. Daughter seed produced by the transgenic plant according to claim 8.

26. A plant as claimed in claim 1 which is dicotyledonous.

27. A plant as claimed in claim 1 which is monocotyledonous.

28. A plant as claimed in claim 1 which is of the family *Gramineae*.

29. A hybrid plant of which at least one parent is a plant according to claim 1.

30. Daughter seed produced by a plant according to claim 1.

31. Non-germinating grain produced from a plant according to claim 1.

32. An isolated, purified nucleic acid sequence encoding phytate produced according to a method comprising:

crossing a low-phytate homozygous mutant plant with a population of plants containing a mutator;

identifying mutator-tagged daughter seed which contain a low-phytate content; and

cloning and isolating said nucleic acid sequence from said daughter seed.

Fig. 1

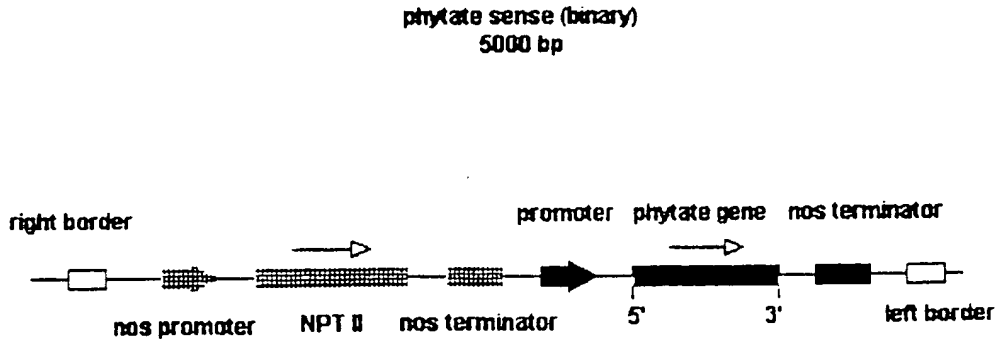
phytate sense (binary)
5000 bp
right border
promoter
phytate gene
nos terminator
nos promoter
NPT II
nos terminator
5'
3'
left border

Fig. 2

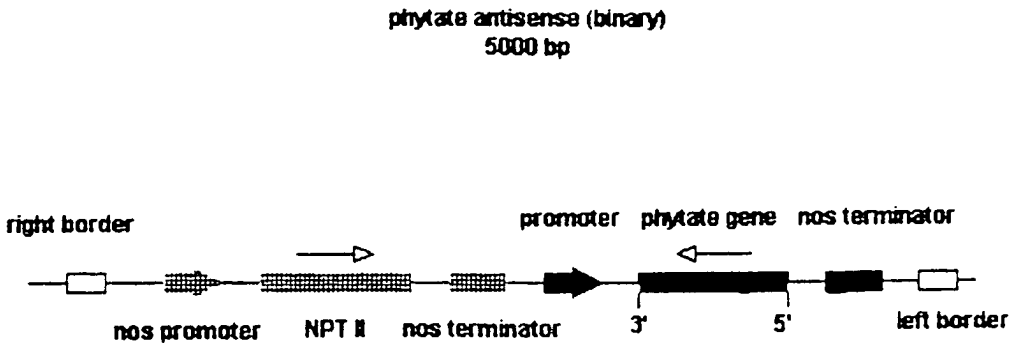

Fig. 3

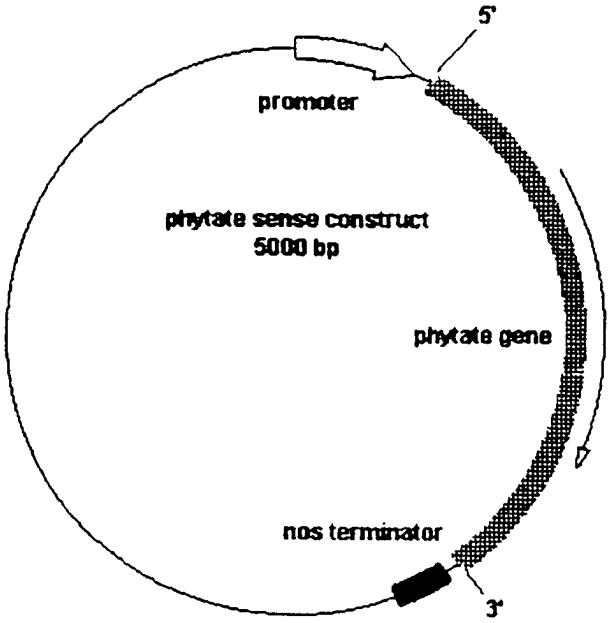

Fig. 4

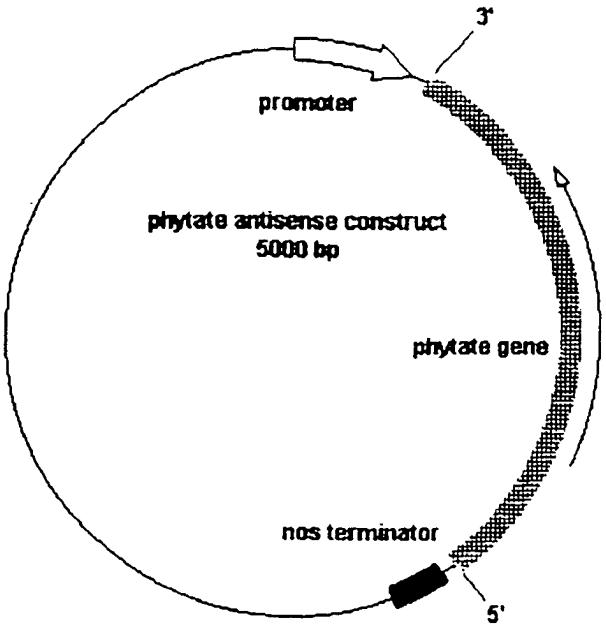

Fig. 5

(i i) MOLECULE TYPE: DNA (genomic)

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ACTGAAGAAT GATGAGTGAC TCACAAAATG GTTTCCCATT GTGGATCAAG AATGGGATTT   60
TCTTGTGAAT TGGGTTCATT TGTAGGAGCA GAGGACTTTT GATCCTCAAG TCCTCCTTCC  120
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TTGTATTCAT AATGAATTCC TTTTTCAGCC AGGGCAATCC TGACCCTCAT CCCAAACATA  180
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AGTATAATGG AATTCCAACA AATATAAAAC TAGAATCAGT TATTATTCAA CATAAACCCA  300
TGAAGTACCA AATTTGTGGG GGTAGAGAGA AGATTTGGAT CGACTAAAAT TTTGACTAGT  360
AAGTTAAAAA AATTAAGGAA CAGAAGAAAG TGGAGCCTTC TTGCTTAACG TTTACTACTA  420
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CTCCCGGACC ACAACACCGC TTGTACCCAT AATAAAAATG TTTAAAAATG AAGACATCTA  540
AGTTTCTACT GGTCTATATA TAGAACTTGA ACTATATACG AAGCATATCA GTTCTAAGCA  600
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Fig. 6

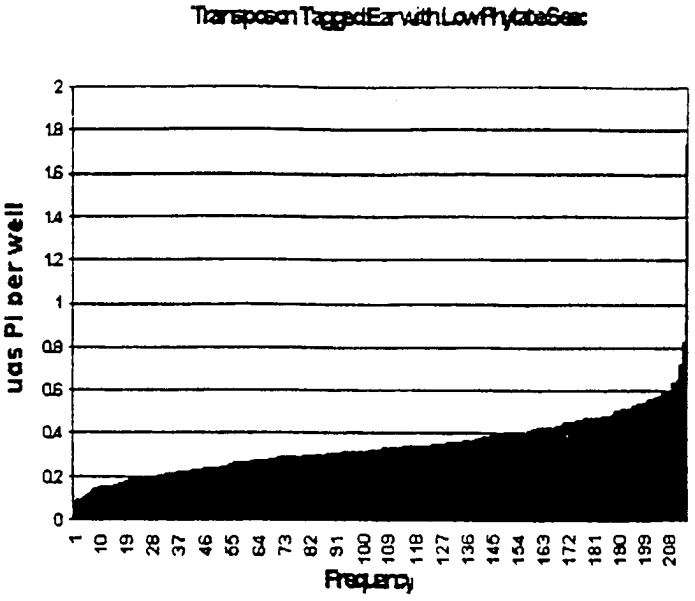

Fig. 7

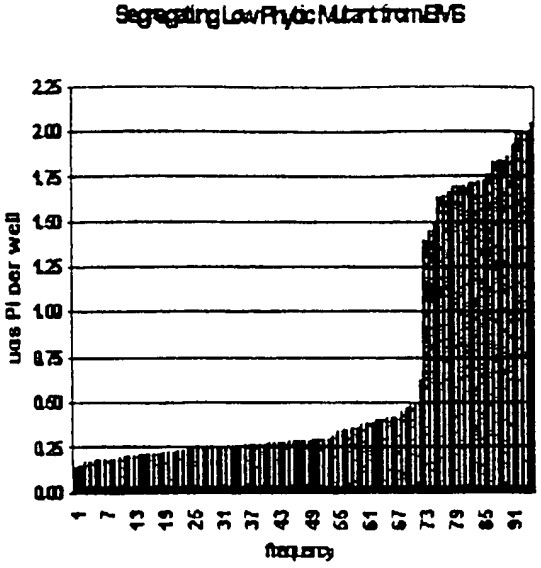
Segregating Low Phytic Mutant from EMS
ug/g Pi per well
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1
7
13
19
25
31
37
43
49
55
61
67
73
79
85
91
frequency

Fig. 8-1

LOCUS AF056326 1665 bp mRNA PLN 03-MAY-1998
DEFINITION Zea mays myo-inositol 1-phosphate synthase mRNA, complete cds.
ACCESSION AF056326
NID g3108052
KEYWORDS
SOURCE Zea mays.
ORGANISM Zea mays
Eukaryota; Viridiplantae; Charophyta/Embryophyta group;
Embryophyta; Tracheophyta; seed plants; Magnoliophyta; Liliopsida;
Poales; Poaceae; Zea.
REFERENCE 1 (bases 1 to 1665)
AUTHORS Larson,S.R. and Raboy,V.
TITLE Linkage mapping maize and barley myo-inositol 1-phosphate synthase genes
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1665)
AUTHORS Larson,S.R. and Raboy,V.
TITLE Direct Submission
JOURNAL Submitted (30-MAR-1998) National Small Grains Germplasm Reserach Facility, USDA-ARS, 1691 South 2700 West, Aberdeen, ID 83210, USA
FEATURES Location/Qualifiers
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RPKSVKYNFRTRTAVPKLGVMLVGWGGNNGSTLTAGVIANREGISWATKDKVQQA
NY

Fig. 8-2

YGSLTQASTIRVGSYNGEEIYAPFKSLLPMVNPDDIVFGGWDISNMNLADSMTRAKVLD

IDLQKQLRPYMESMVPLPGIYDPDFIAANQGSRANSVIKGTKKEQVEQIIKDIREFKE

KNKVDKIVVLWTANTERYSNVCAGLNDTMENLLASVDKNEAEVSPSTLYAIACVMEG
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Fig. 9-1

(Linear) MAP of Zea Myo-inositol 1-phosphate synthase from: 86 to: 1620

```
          atgttcatcgagagcttccgcgtcgagagccccccacgtgcggtacggcccgatggagatc
       86 ----+---------+---------+---------+---------+---------+----- 145
          tacaagtagctctcgaagggcgcagctctcgggggtgcacgccatgccgggctacctctag

a         M  F  I  E  S  F  R  V  E  S  P  H  V  R  Y  G  P  M  E  I  -

          gagtcggagtaccggtacgacacgacggagctggtacacgagggcaaggacggcgcctca
      146 ----+---------+---------+---------+---------+---------+----- 205
          ctcagcctcatggccatgctgtgctgcctcgaccatgtgctcccgttcctgccgcggagt

a         E  S  E  Y  R  Y  D  T  T  E  L  V  H  E  G  K  D  G  A  S  -

          cgctgggtcgtccgccccaagtccgtcaagtacaacttccggaccagaaccgccgtcccc
      206 ----+---------+---------+---------+---------+---------+----- 265
          gcgacccagcaggcggggttcaggcagttcatgttgaaggcctggtcttggcggcagggg

a         R  W  V  V  R  P  K  S  V  K  Y  N  F  R  T  R  T  A  V  P  -

          aagctcggggtgatgcttgtggggtggggaggcaacaacgggtccacgctgacggctggg
      266 ----+---------+---------+---------+---------+---------+----- 325
          ttcgagccccactacgaacaccccaccctccgttgttgcccaggtgcgactgccgaccc

a         K  L  G  V  M  L  V  G  W  G  G  N  N  G  S  T  L  T  A  G  -

          gtcattgccaacagggaggggatctcatgggcgaccaaggacaaggtgcagcaagccaac
      326 ----+---------+---------+---------+---------+---------+----- 385
          cagtaacggttgtccctccccctagagtacccgctggttcctgttccacgtcgttcggttg

a         V  I  A  N  R  E  G  I  S  W  A  T  K  D  K  V  Q  Q  A  N  -

          tactacggctccctcacccaggcctccaccatcagagtcggcagctacaacggggaggag
      386 ----+---------+---------+---------+---------+---------+----- 445
          atgatgccgagggagtgggtccggaggtggtagtctcagccgtcgatgttgccccctcctc

a         Y  Y  G  S  L  T  Q  A  S  T  I  R  V  G  S  Y  N  G  E  E  -

          atctatgcgccgttcaagagcctccttcccatggtgaacccagacgacattgtgttcgga
      446 ----+---------+---------+---------+---------+---------+----- 505
          tagatacgcggcaagttctcggaggaagggtaccacttgggtctgctgtaacacaagcct

a         I  Y  A  P  F  K  S  L  L  P  M  V  N  P  D  D  I  V  F  G  -

          ggctgggacattagcaacatgaacctggccgactccatgaccagggccaaggtgctggat
      506 ----+---------+---------+---------+---------+---------+----- 565
          ccgaccctgtaatcgttgtacttggaccggctgaggtactggtcccggttccacgaccta

a         G  W  D  I  S  N  M  N  L  A  D  S  M  T  R  A  K  V  L  D  -

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      566 ----+---------+---------+---------+---------+---------+----- 625
          taactggacgtcttcgtcgagtccgggatgtacctcaggtaccacggtgaagggccatag

a         I  D  L  Q  K  Q  L  R  P  Y  M  E  S  M  V  P  L  P  G  I  -

          tatgatccggacttcatcgcggctaaccagggctctcgcgccaacagtgtcatcaagggc
      626 ----+---------+---------+---------+---------+---------+----- 685
          atactaggcctgaagtagcgccgattggtcccgagagcgcggttgtcacagtagttcccg
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Fig. 9-2

```
a       Y  D  P  D  F  I  A  A  N  Q  G  S  R  A  N  S  V  I  K  G  -

        accaagaaagaacaggtggagcagatcatcaaggatatcagggagtttaaggagaagaac
686     ----+---------+---------+---------+---------+---------+----- 745
        tggttctttcttgtccacctcgtctagtagttcctatagtccctcaaattcctcttcttg

a       T  K  K  E  Q  V  E  Q  I  I  K  D  I  R  E  F  K  E  K  N  -

        aaagtggacaagatagttgtgttgtggactgcaaacactgaaaggtatagcaatgtgtgc
746     ----+---------+---------+---------+---------+---------+----- 805
        tttcacctgttctatcaacacaacacctgacgtttgtgactttccatatcgttacacacg

a       K  V  D  K  I  V  V  L  W  T  A  N  T  E  R  Y  S  N  V  C  -

        gctggtctcaacgacacgatggagaatctactggcatctgtggacaagaacgaggcggag
806     ----+---------+---------+---------+---------+---------+----- 865
        cgaccagagttgctgtgctacctcttagatgaccgtagacacctgttcttgctccgcctc

a       A  G  L  N  D  T  M  E  N  L  L  A  S  V  D  K  N  E  A  E  -

        gtatcaccatcaatcttatatgccattgcctgtgtcatggaggggtgccgttcatcaat
866     ----+---------+---------+---------+---------+---------+----- 925
        catagtggtagttgtgatatacggtaacggacacagtacctccccacggcaagtagtta

a       V  S  P  S  T  L  Y  A  I  A  C  V  M  E  G  V  P  F  I  N  -

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926     ----+---------+---------+---------+---------+---------+----- 985
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a       G  S  P  Q  N  T  F  V  P  G  L  I  D  L  A  I  K  N  N  C  -

        ttgattggtggtgacgacttcaagagtggacagaccaagatgaaatctgtcttggtcgat
986     ----+---------+---------+---------+---------+---------+----- 1045
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1046    ----+---------+---------+---------+---------+---------+----- 1105
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1166    ----+---------+---------+---------+---------+---------+----- 1225
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a       N  V  V  D  D  M  V  S  S  N  A  I  L  Y  E  P  G  E  H  P  -

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1226    ----+---------+---------+---------+---------+---------+----- 1285
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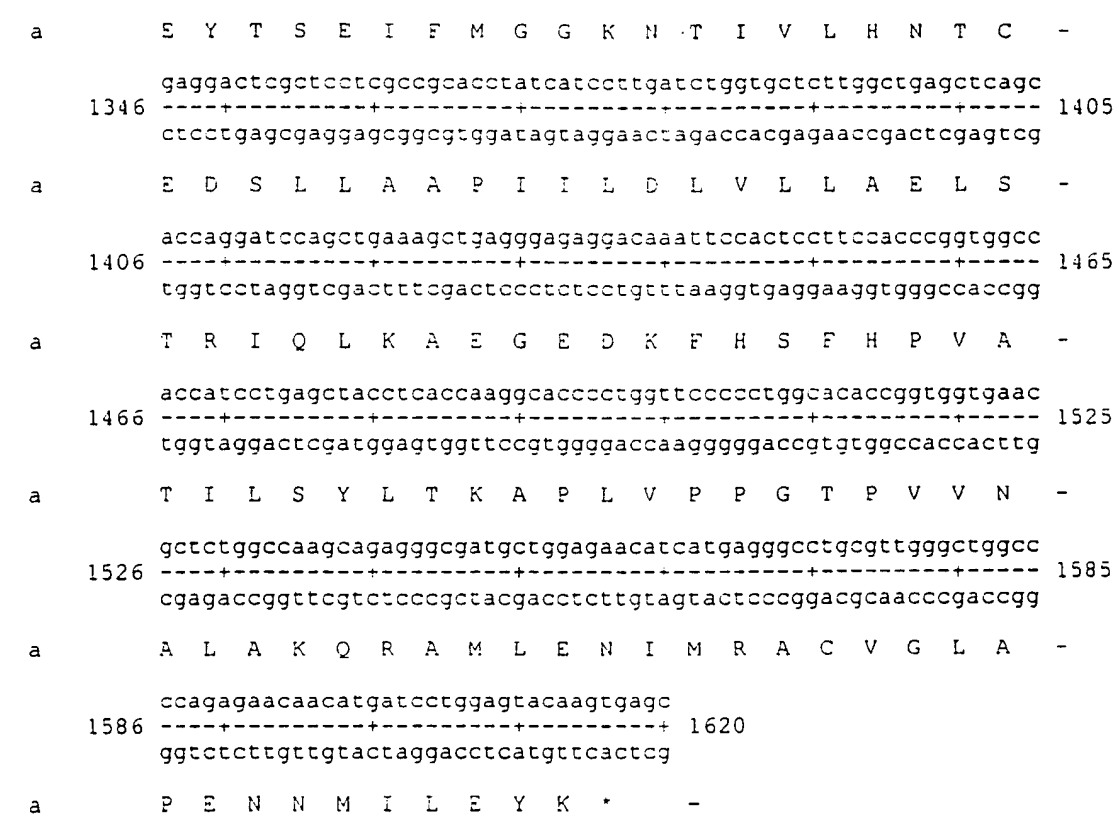
Fig. 9-3



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Guan, Hanping
Chang, Ming-Tang
Wilhelm, Edward P.

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His Glu Gly Lys Asp Gly Ala Ser Arg Trp Val Val Arg Pro Lys Ser
35 40 45

Val Lys Tyr Asn Phe Arg Thr Arg Thr Ala Val Pro Lys Leu Gly Val
50 55 60

Met Leu Val Gly Trp Gly Gly Asn Asn Gly Ser Thr Leu Thr Ala Gly
65 70 75 80

Val Ile Ala Asn Arg Glu Gly Ile Ser Trp Ala Thr Lys Asp Lys Val
85 90 95

Gln Gln Ala Asn Tyr Tyr Gly Ser Leu Thr Gln Ala Ser Thr Ile Arg
100 105 110

Val Gly Ser Tyr Asn Gly Glu Glu Ile Tyr Ala Pro Phe Lys Ser Leu
115 120 125

Leu Pro Met Val Asn Pro Asp Asp Ile Val Phe Gly Gly Trp Asp Ile
130 135 140

Ser Asn Met Asn Leu Ala Asp Ser Met Thr Arg Ala Lys Val Leu Asp
145 150 155 160

Ile Asp Leu Gln Lys Gln Leu Arg Pro Tyr Met Glu Ser Met Val Pro
165 170 175

Leu Pro Gly Ile Tyr Asp Pro Asp Phe Ile Ala Ala Asn Gln Gly Ser
180 185 190

Arg Ala Asn Ser Val Ile Lys Gly Thr Lys Lys Glu Gln Val Glu Gln
195 200 205

Ile Ile Lys Asp Ile Arg Glu Phe Lys Glu Lys Asn Lys Val Asp Lys
210 215 220

Ile Val Val Leu Trp Thr Ala Asn Thr Glu Arg Tyr Ser Asn Val Cys
225 230 235 240

Ala Gly Leu Asn Asp Thr Met Glu Asn Leu Leu Ala Ser Val Asp Lys
245 250 255

Asn Glu Ala Glu Val Ser Pro Ser Thr Leu Tyr Ala Ile Ala Cys Val
260 265 270

Met Glu Gly Val Pro Phe Ile Asn Gly Ser Pro Gln Asn Thr Phe Val
275 280 285

Pro Gly Leu Ile Asp Leu Ala Ile Lys Asn Asn Cys Leu Ile Gly Gly
290 295 300

Asp Asp Phe Lys Ser Gly Gln Thr Lys Met Lys Ser Val Leu Val Asp
305 310 315 320

Phe Leu Val Gly Ala Gly Ile Lys Pro Thr Ser Ile Val Ser Tyr Asn
325 330 335

His Leu Gly Asn Asn Asp Gly Met Asn Leu Ser Ala Pro Gln Ala Phe
340 345 350

Arg Ser Lys Glu Ile Ser Lys Ser Asn Val Val Asp Asp Met Val Ser
355 360 365

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Val Ile Lys Tyr Val Pro Tyr Val Gly Asp Ser Lys Arg Ala Met Asp
385                 390                 395                 400

Glu Tyr Thr Ser Glu Ile Phe Met Gly Gly Lys Asn Thr Ile Val Leu
                405                 410                 415

His Asn Thr Cys Glu Asp Ser Leu Leu Ala Ala Pro Ile Ile Leu Asp
            420                 425                 430

Leu Val Leu Leu Ala Glu Leu Ser Thr Arg Ile Gln Leu Lys Ala Glu
        435                 440                 445

Gly Glu Asp Lys Phe His Ser Phe His Pro Val Ala Thr Ile Leu Ser
    450                 455                 460

Tyr Leu Thr Lys Ala Pro Leu Val Pro Pro Gly Thr Pro Val Val Asn
465                 470                 475                 480

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INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US98/16702

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/250; 435/068.1, 069.1, 094, 195, 200, 204, 209, 232, 233, 234; 426/007, 061, 531, 635.

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